STUDIES ON ANTIGENS OF ASPERGILLUS; THEIR USE IN VETERINARY MYCOLOGY

BY

ROBERTO ARNULFO CERVANTES-OLIVARES, M.V.Z.

Thesis submitted for the degree of Doctor of Philosophy in the Faculty of Veterinary Medicine, the University of Glasgow.

Department of Veterinary Pathology,
University of Glasgow.

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CONTENTS

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SUMMARY

Five strains of Aspergillus fumigatus, 4 wild-type and one morphologically abnormal, were used to produce antigens. They were cultured on Glucose peptone and Yeast malt (Difco) at 28, 37 and 46°C and were harvested weekly from 1 - 6 weeks. All antigens were standardised on the dry weight of the mycelium and were tested against 3 sera.

Both media gave good antigens; the protein content was higher in antigens produced on Y.M. although the weight of the mycelium was lower. The weight of mycelium varied with the strain and increased as the incubation temperature was raised. Temperature also affected antigenicity; 28 and 37°C were suitable but 46°C gave less good antigens.

Ability to react in serological tests varied; The best antigens were culture filtrate antigens from G. P. at 37°C of the abnormal strain. Mycelial antigens gave better results in Double diffusion tests and culture filtrate antigens in Counterimmunoelectrophoresis. Overall, D.D. tests showed a higher percentage of positive tests than C.I.E. The reaction of the antigens also varied with the serum used.

The factors involved in the production of fungal exudates were investigated and exudates from species of Aspergillus were collected. These proved to be excellent antigens.

An 18-hour germinating spore antigen of A. fumigatus was prepared in dialysate of bovine allantoic fluid in sterile water. In C.I.E. tests this gave better results than other antigens with sera from cases

of bovine mycotic abortion.

It has been shown that serological diagnosis of mycotic abortion is not possible; some sera from proven A. fumigatus cases were positive but others were negative while sera from other types of mycotic abortion and from normal cattle were positive.

Serological surveys proved that antibody to A. fumigatus was present in sera from normal cats and dogs. A second survey of dogs in which the nasal passages and ears were examined at autopsy and by culture for fungi proved that antibody occurs in sera from healthy dogs. That otitis externa caused by Pityrosporum pachydermatis can cause production of serum antibody was shown for the first time and the possibility of cross-reactions with this and A. fumigatus in D.D. tests was shown to be unlikely.

The reliability of laboratory methods of diagnosis of canine nasal aspergillosis was investigated and it was found that the only samples giving reliable results were swabs taken directly from the lesion or tissues removed at operation.

Cases of nasal aspergillosis were followed. Treatment trials with ketoconazole resulted in 1 cure and 3 failures. Sections of lesions showed that the fungus was embedded in necrotic tissue which may explain the failure of treatment. Titration of serum was found to be the best laboratory method of assessing the progress of the dogs.

INTRODUCTION - GENERAL

The genus Aspergillus was named by Micheli in 1729 who described 9 species. Fungi in this genus were also noted from decaying vegetation by Link (1809) but it was not until about 1850 when de Bary and others began laboratory cultivation that descriptions were such that the fungus involved was identifiable by other workers.

Systematic study of the genus Aspergillus really commenced with Thom and his co-workers who investigated species of Aspergillus responsible for deterioration of foodstuffs. The culmination of this work was "The Aspergilli" published in 1926 by Thom and Church, followed in 1945 by "A Manual of the Aspergilli" by Thom and Raper and in 1965 by "The Genus Aspergillus" by Raper and Fennell. The most recent publication is "Genetics and Physiology of Aspergillus", a British Mycological Society Symposium Series published in 1977.

Aspergilli are among the most ubiquitous and adaptable of the fungi and can affect man and animals in many ways. On the credit side, Aspergillus spp. are used in industrial processes such as the production of citric acid and of industrial enzymes (Smith, 1946). On the debit side, however, species of Aspergillus are of major importance in the spoilage of food and not only is food spoiled, but certain Aspergillus spp. growing on foodstuffs produce toxins which on ingestion cause mycotoxicoses, of which aflatoxicosis is probably the best known example (Wyllie and Morehouse, 1978). Furthermore, some species with a tendency to proliferate on certain

substrates can act as allergens, mainly affecting the respiratory tract. A well-known example of allergic aspergillosis is Malt Worker's Lung, an extrinsic allergic alveolitis caused by inhalation of and sensitization to A. clavatus or A. fumigatus growing on malting barley (Pepys, 1969). However, the study of Aspergillus spp. as toxin and allergen producers is in its infancy in comparison with that of those species which can produce disease by invasion of the tissues of the living body.

The infection characterised by invasion of living tissues by fungi of the genus Aspergillus is known as aspergillosis. The infection can be primary, when it is the result of the direct entry of the fungus into a healthy susceptible organ of the body; or secondary, when the growth of the fungus is aided by predisposing factors e.g. wounds, debilitating disease or antibiotic or corticosteroid therapy (Austwick, 1965).

In man, one of the first identifiable cases of aspergillosis was reported by Virchow (1856) but it was Kenon's classic paper in 1897, which stimulated great interest in the disease (Emmons, Binford and Utz, 1970). Aspergillosis in humans has been described from almost every part of the body but it is usually a lung infection which, depending on the immunological status of the person, can be localised and controlled or it can be invasive and destructive.

Landau, Newcomer and Schulz (1963) reported the condition in

an 18 day old child and in an 86 year old man, so age does not seem to be an important factor, nor does sex or race. Aspergillosis is a growing problem according to Fraser, Ward, Ajello and Plikaytis (1979); they said that in the U. S. A. the incidence of the disease rose sharply from 1970 to 1976. One of the factors involved with the high incidence has been the development and use of antibacterial antibiotics, especially those with a broad spectrum of action. Other drugs such as corticosteroids, immunosuppressive and cytotoxic drugs have played an important role in this as well (Finegold, Will and Murray, (1959).

Aspergillosis in man has been extensively studied and described and the literature has been reviewed by Finegold et al (1959), Landau, Newcomer and Schultz (1963), Symmers (1962) and Chandler, Kaplan and Ajello (1980). The most recent review is that of Bardana (1981) which also includes references to aspergillosis in animals and birds.

According to Ainsworth and Austwick (1973) avian aspergillosis is world-wide in distribution and occurs in all species of domestic poultry and in many species of wild birds. Avian aspergillosis is probably the only aspergillosis in the veterinary field in which the incidence of the disease has been reduced. This was achieved by prevention of infection by improved husbandry and hygiene. The bibliographies of Chute, O'Meara and Barden (1962), Barden, Chute, O'Meara and Wheelwright (1971) and Bardana (1981) cite most of

the papers on the subject.

In the horse, although pulmonary aspergillosis has been reported (Long and Mitchell, 1971) and cases of rhinitis caused by A. fumigatus occur (Greet, 1981), in most cases of equine aspergillosis it is the guttural pouches which are involved. These structures, peculiar to equines, are thin-walled mucous sacs each of which is a diverticulum of the Eustachian tube. The pouches lie between the base of the cranium and the pharynx and are in close apposition to the internal carotid artery and to the glossopharyngeal, vagus and spinal accessory nerves. Spread of the fungus, which may be A. fumigatus or A. nidulans, to the nerves causes laryngeal and pharyngeal paralysis which may cause aspiration pneumonia and spread to the artery may cause death by haemorrhage. The main clinical features of the disease were described by Cook (1968) and the pathology and aetiology by Cook, Campbell and Dawson (1968) who also reviewed the literature on the subject. Cases of mycotic abortion, some caused by A. fumigatus, have occurred in mares (Mahaffey and Adam, 1964; Mahaffey and Rossdale, 1965).

Cattle, however, are the animals most prone to mycotic abortion which, in temperate zones is a disease of considerable economic importance. Of the many species of fungi which can cause this condition, A. fumigatus is one of the most important. The first record of this species as the cause of bovine mycotic abortion was,

according to Francalanci (1959), that of Bendixen and Plum in 1929. Weidlich (1952) isolated A. fumigatus from the skin of an aborted foetus and the same species was reported by Ainsworth and Austwick (1955) as the causative agent in 63% of 51 mycotic abortion cases. In Italy, Ballarini (1955) isolated this species from 4 of his 5 cases and in Portugal, Moreira-Jacob and van Uden (1956) isolated A. niger from an aborted foetus. Francalanci(1959) reported 15 cases of A. fumigatus abortion from Italy and he also studied the incidence of Aspergillus spp. on the prepuce and in semen of healthy bulls and suggested that genital tract infection was responsible for mycotic abortion.

The studies of Bendixen and Plum (1929) on mycotic abortion led them to believe that the primary site of infection was the lung and that the fungus spread to the placenta through the blood stream. Circumstantial evidence supports this theory; more cases of the disease occur in winter (Ainsworth and Austwick, 1955) and there is a statistical relationship between the number of rain days in June and the number of infections in the following winter (Hugh-Jones and Austwick, 1967). Hillman and McEntee (1969) failed to induce abortion by introduction of spores into the trachea but after challenge by the intravenous route, abortion occurred.

The pathogenesis of mycotic abortion was studied by Hill, Whiteman, Benjamin and Ball (1971). They described necrotising lesions in the placentomes and extensive involvement of the inter-

placentomal area which supported their theory that infection spread from the arcade zone of the placentome to the other tissues.

An important advance in the study of mycotic abortion due to A. fumigatus was made by White and Smith (1974) when they found that extracts from foetal tissues enhanced germination of the spores. They found that the best extracts were obtained from cotyledon and from allantoic fluid. I used this ability of allantoic fluid to enhance germination in the preparation of an antigen from germinating spores of A. fumigatus.

Bovine aspergillosis is not only a disease of the reproductive system. Pneumonia has been reported by Eggert and Romberg (1960) and by Cordes, Dodd and O'Hara (1964). The post mortem findings were of an acute or necrotising pneumonia with septate hyphae being present in the diseased portions of the lung. Austwick (1962) noted minute lung lesions which, in section, showed hyphae surrounded by a sheath of strongly eosinophilic material in club formation. He called these "asteroid bodies" and suggested that they were a manifestation of resistance to infection. Similar structures, "rosettes," had been noted by Davis and Schaefer (1962), in a case of cutaneous aspergillosis in a cow, caused by A. terreus.

In a report of pulmonary aspergillosis in lambs, caused by A. fumigatus, Austwick, Gitter and Watkins (1960) described different types of hyphal morphology associated with the stage of the infection:

swollen hyphal cells characteristic of the acute stage, closely branched actinomycetoid hyphae as the infection developed and, in the chronic stage, hyphae resembling those found when the fungus is grown in submerged culture. Pulmonary aspergillosis in lambs has also been described by Ayaz, Ilahi and Afzal (1966) and by Young (1970) and a case of generalised aspergillosis by Gracey and Baxter (1961). A case of infection of the nasal passages of a sheep which was probably infested by Oestrus ovis, the larvae of the sheep nasal fly, was reported by Austwick in 1966. The causal fungus was A. fumigatus.

Many workers have used sheep as an experimental model to study mycotic abortion and a number of reports of experimental mycotic abortion in ewes appear in the literature. They describe abortion induced by intravascular and intrauterine inoculation (Pier, Cysewski and Richard, 1972), pathological changes (Cysewski and Pier, 1968), haematological changes (Day and Corbel, 1974), serological response (Corbel, Pepin and Millar, 1973) and examination of the immunoglobulin classes involved (Corbel and Day, 1978). Recently, Corbel, Day and Cole, (1980) reported on the relationship between pathological changes, immunological response and serum protein concentration in pregnant ewes inoculated with A. fumigatus. Natural ovine mycotic abortion has not, so far, been reported.

Aspergillosis is not restricted to farm stock but also occurs in companion animals. The first report of aspergillosis in the cat

was that of Sautter, Steele and Henry (1955) who described pulmonary involvement. This was later confirmed by the cases described by Pakes, New and Benbrook (1967) and McCausland (1972). Infection of the digestive tract was reported by Bolton and Brown (1972) and Stokes (1973). Cases of involvement of both systems were described by Vogler and Wagner (1975) and Fox, Murphy and Shalev (1978). Recently, Wilkinson, Sutton and Grono (1982) reported a completely different syndrome caused by an Aspergillus sp. in a cat suffering from proptosis of the eyeballs, prolapse of the membrana nictitans and frontal sinusitis. This case is interesting in regard to comparison with canine nasal aspergillosis which is mainly a nasal and sinus infection. This will be discussed in detail later.

Although there is a report of pulmonary aspergillosis in a dog (Ohshima,Naito and Seimiya, 1979), no case of infection of the digestive tract has been reported. A lesion in the central nervous system was noted by Parker and Cunningham (1971), keratoconjuntivitis (Schmidt, 1974), destruction of vertebrae (Weitkamp, 1982), and generalised invasion in a severely ill dog (Isoun, 1975).

The diagnosis of aspergillosis in animals lags far behind that in man where serological tests are extensively used as an adjunct to clinical diagnosis. Pepys, Ridell, Citron, Clayton and Short (1959) used double diffusion to investigate sera from cases of respiratory disorders; Dee (1975) used counterimmunoelectrophoresis; Biguet, Tran van Ky, Fruit and Andrieu (1964) used immunoelectrophoresis

and Hipp, Berns, Tompkins and Buckley (1970) latex agglutination. More sophisticated tests have been developed e.g. radioallergosorbent test has been used by Arbesman, Wicher, Wypych, Reisman, Dickie and Reed (1974) and the enzyme linked immunosorbent assay by Sepulveda, Longbottom and Pepys (1979).

Serological tests have been little used in the diagnosis of aspergillosis in animals. Corbel (1972) tested sera from normal cows and from cases of mycotic abortion by double diffusion using an antigen prepared from mycelium of A. fumigatus and stated that precipitins were infrequent in sera from normal cattle but were present in most sera from abortion cases in which species of Aspergillus had been implicated. The most successful use of a serological test is in the diagnosis of canine nasal aspergillosis (Lane and Warnock, 1977).

MATERIALS AND METHODS

The following nutrient media were used in the production of antigens, in the study of exudates from Aspergilus spp. and for isolation of fungi from normal and pathological specimens.

Czapek-Dox Agar (Raper and Fennell, 1965).

Sodium nitrate $NaNO_3$	3.0g
Di-potassium hydrogen phosphate K_2HPO_4	1.0g
Magnesium sulphate $MgSO_4.7H_2O$	0.5g
Potassium chloride KCl	0.5g
Ferrous sulphate $FeSO_4.7H_2O$	0.01g
Sucrose	30.0g
Agar Technical No.3	12.0g
De-ionised water	1000 ml

The reagents were dissolved in the water and boiled to dissolve the agar, sucrose was then added and the medium sterilised by autoclaving at 121°C for 20 min., poured in 20ml amounts into 8.5cm Petri dishes or in 10ml amounts into universal bottles.

Czapek-Dox Agar (Modified) Oxoid

Sodium nitrate $NaNO_3$	2.0g
Potassium chloride KCl	0.5g
Magnesium glycerophosphate $MgC_3H_5(OH)_2PO_4.H_2O$	0.5g
Ferrous sulphate $FeSO_4.7H_2O$	0.01g
Potassium sulphate K_2SO_4	0.35g
Sucrose	30.0g
Agar Technical No.3	12.0g

33.4g of the dehydrated medium were dissolved in 1 litre of de-ionised water, distributed into 3XVI Saniglass bottles in 100ml amounts and sterilised by autoclaving at 115°C for 20 min. pH unadjusted 6.8.

Glucose Peptone Liquid Medium

D-glucose (BDH)	40.0g
Mycological peptone (Oxoid)	10.0g
De-ionised water	1000ml

The ingredients were dissolved, distributed in 100ml lots into bottles and sterilised by autoclaving at 115°C for 20 min. pH unadjusted 5.8

1% Glucose Peptone Liquid Medium

D-glucose (BDH)	10.0g
Mycological peptone (Oxoid)	10.0g
De-ionised water	1000ml

Preparation as above.

Glucose Peptone agar

D-glucose (BDH)	40.0g
Mycological peptone (Oxoid)	10.0g
Agar Technical No. 3	12.0g
De-ionised water	1000 ml

The agar was dissolved in water by steaming and the glucose and peptone added and the medium was sterilised by autoclaving at 115°C for 20min. and distributed in 20ml amounts into 8.5cm Petri dishes. pH unadjusted 5.8.

Glucose Peptone Agar with Chloramphenicol

10ml of a solution of 0.5g chloramphenicol in 100ml of 95% ethyl alcohol were added to the glucose peptone agar before sterilisation by autoclaving at 115°C for 20 min.

2% Malt Extract Agar with Chloramphenicol

Malt Extract (D.C.L.)	20g
Agar Technical No. 3	12g
De-ionised water	1000ml
Chloramphenicol (0.5g in 100ml alcohol)	10ml

The agar was dissolved in water by steaming, the malt was dissolved in the molten agar and the chloramphenicol solution added before sterilisation by autoclaving at 115°C for 15 min. and distributed in 20ml amounts into 8.5cm Petri dishes.

4% Malt Extract Agar

Malt Extract (D.C.L.)	40g
Agar Technical No. 3	12g
De-ionised water	1000ml

Preparation as above.

4% Malt Extract Agar with Chloramphenicol

10ml of a solution of 0.5g chloramphenicol in 100ml ethyl alcohol were added to the medium before sterilising by autoclaving at 115°C for 15 min.

Yeast Malt Liquid Medium (Difco)

Bacto Yeast Extract	3.0g
Bacto Malt Extract	3.0g
Bacto Peptone	5.0g
Bacto Dextrose	10.0g

41.0g of dehydrated medium were dissolved in 100ml de-ionised water, distributed in 100ml lots into bottles and sterilised by autoclaving at 121°C for 15 min.

Yeast Nitrogen Base Agar (Difco)

Bacto Yeast Nitrogen base	117.0g
Agar Technical No. 3	12.0g
De-ionised water	1000ml

The dehydrated medium was suspended in water, the agar added and dissolved by steaming. Amounts of 10ml were put into universal bottles and sterilised by autoclaving at 115°C for 20 min. pH 5.3 unadjusted.

This medium was supplemented with 10%, 20% or 30% of glucose or sucrose or with 10% mannitol.

Buffers and Reagents

Phosphate Buffered Saline

Sodium chloride NaCl	8.0g
Di-potassium hydrogen phosphate K_2HPO_4	1.21g
Potassium dihydrogen phosphate KH_4PO_4	0.54g
De-ionised water	1000ml

The ingredients were suspended in the water and stirred until dissolved. pH 7.2.

Borate Buffer

Boric acid H_3BO_3	7.7g
Di-sodium tetraborate $Na_2B_4O_7.10H_2O$	13.4g
De-ionised water	500ml

The water was heated to 70°C and the ingredients dissolved by stirring. pH 8.6

Veronal (Barbitone) Buffer

Diethyl barbituric acid (Barbitone) $(C_2H_5)_2C.CO.NH.CO.NH.CO$	3.32g
Sodium diethyl barbitone (Barbitone sodium) $(C_2H_5)_2C.CO.NH.C.CONa$	25.52g
Sodium azide NaN_3	2.0g
De-ionised water	2000ml

The Barbitone was dissolved in 200ml hot de-ionised water, the Barbitone sodium was dissolved and the solutions mixed and made up to 2000ml. Sodium azide was added as a preservative.

Coca's Saline

Sodium chloride NaCl	5.0g
Sodium bicarbonate $NaHCO_3$	2.75g
Phenol crystals C_6H_5OH	4.0g
De-ionised water	1000ml

The ingredients were dissolved and the solution was sterilised by autoclaving at $121^{o}C$ for 15 min.

Merthiolate Solution

Thiomerosal $C_2H_5HgSC_6H_4COONa$	1g
De-ionised water	100ml

5% Sodium citrate Solution

Trisodium citrate $Na_3C_6H_5O_7.2H_2O$	5.0g
De-ionised water	100ml

Gel de-staining Fluid

Methanol	500ml
Glacial acetic acid	100ml
De-ionised water	400ml

The ingredie ts were mixed and stored in an amber bottle.

Amido Black Stain

Amido (naphthalene) black	1g
Methanol-glacial acetic acid-water	1000ml

20% Potassium hydroxide (KOH) solution

Lactophenol Cotton Blue

Gomori methenamine silver nitrate stain

(Grocott modification for fungi)

Gram stain

Periodic acid-Schiff stain

Haematoxylin and Eosin stain

Serological methods

Borate Buffered Agar (Murray and Mahgoub,1968)

Borate buffer	100ml
Ion agar No. 2 (Oxoid)	3.0g
De-ionised water	100ml
Sodium azide NaN_3	0.2g

The agar was heated in the water until it was dissolved and the buffer, heated to 56°C in a water bath, added as was Sodium azide. The mixture was poured in 10ml amounts into 8.5cm plastic Petri dishes on a levelling table and allowed to set, then stored at 4°C.

Double diffusion (D.D.) (Ouchterlony) test

All D.D. tests were run in Borate buffered agar. A number of

well patterns were used (Fig. 1).

A. A 6mm central well surrounded by 4 3mm wells, with a distance of 6mm edge to edge between the central and peripheral wells

B. A 6mm central well surrounded by 5 3mm wells, with a distance of 6mm edge to edge between the central and peripheral wells.

C. A 6mm central well surrounded by 6 3mm wells, with a distance of 6mm edge to edge between the central and peripheral wells.

D. A 3mm central well surrounded by 6 6mm wells, with a distance of 6mm edge to edge between the central and peripheral wells.

A template was drawn on graph paper for each well pattern. This was placed beneath the Petri dish and the wells were cut with steel cork-borers. A bent needle was used to remove the agar. The wells were filled, using Pasteur pipettes and the plates were put into a humid chamber for 7 days and examined daily for precipitation lines. After the 7 days the plates were flooded with a solution of 5% Sodium citrate for 45 min. to eliminate the possibility of false positives due to C-reactive protein in the antigens. The gels were then removed from the Petri dishes and washed in Phosphate buffered saline for 48 h. at 4°C then for 24 h. in de-ionised water. They were than transferred to 8 x 8cm glass plates and dried at 46°C before staining with 0.1% Amido black and de-staining in gel de-staining fluid. Stained slides (Fig. 2) were kept as permanent records.

Veronal Buffered Agarose

Veronal buffer	100ml
De-ionised water	100ml
Agarose (Miles-Serevac)	2.0g

The agarose was dissolved in the water and the buffer, heated to 56°C in a water bath was added.

Counterimmunoelectrophoresis test (C.I.E.)

Five acid-cleaned glass slides (3.9 x 7.6cm) were used to support the agarose gels. These were fixed to a plastic tray with a small amount of agarose and 40ml of Veronal buffered agarose, pH 8.2, were poured over the slides on the tray which had been placed on a levelling table. After setting, the agarose gel was held at 4°C for 10 min. to firm.

Parallel rows of wells, 3mm in diameter and 5mm edge to edge, were cut using steel cork-borers and the agarose removed from the wells with a bent needle. The wells were filled with the reactants using Pasteur pipettes. The tray was placed in a Shandon electrophoresis tank containing 500ml full strength Veronal buffer per compartment with the serum wells to the anode and the antigen wells to the cathode. The tray was connected to the electrolyte by lint wicks which were gently pressed down to ensure good, even contact between gel and electrolyte. A constant electric current was passed through the gel from a D.C. power unit (Shandon)

at the rate of 2mA per cm width for 50 min. After the run, the slides were cooled at 4°C for 15 min., read for precipitation lines, then carefully removed from the tray to a plastic container containing Phosphate buffered saline. The gels, held on the slides by rubber bands, were washed in P.B.S. for 24 hours then in de-ionised water for 24 hours. After drying at 46°C the slides were stained with 1% Amido black. Stained slides (Fig.3) were examined with a magnifying glass and after being read were kept as permanent records.

Immunoelectrophoresis test (I.M.E.)

Five acid-cleaned slides, 3.9 x 7.6cm, were used to support the gel. These were held in a plastic tray with a small amount of agarose and 40ml of Veronal buffered agarose, pH 8.2, poured over then. After setting, the gel was removed from the levelling table and was put at 4°C for 10 min. to firm. The wells and troughs, 2mm in diam. were cut with a Shandon trough cutter and the agarose in the wells removed by suction. The antigens were put into the wells using capillary tubes and the tray was placed in a Shandon electrophoresis tank containing 500ml full strength Veronal buffer per compartment. The tray was connected to the electrolyte by lint wicks which were gently pressed down to ensure good contact. A constant voltage of 150 volts (7.7 volts per cm.) was applied for 50 min. After the run the gel was removed from the troughs and these were filled with serum. The tray was placed in a humid chamber for up to 3

days to allow the arcs to develop. The gels were washed and stained in the same way as C.I.E. slides and were kept as permanent records.

Antigens

The following antigens were used in diagnostic work and in serological surveys. The first 3 antigens described are those used in routine diagnosis in the laboratory, the remainder were prepared for use in the surveys.

A. fumigatus:

112 (Mycelial extract) The mycelial mat from 2 strains, V215 from a gosling and 1358 from the skin of a horse, cultured on 1% glucose 3% peptone liquid medium in Roux bottles at 28°C for 6 weeks, was extracted with Coca's saline for 10 days at 4°C, dialysed against tap water, concentrated x 10 by ultrafiltration (Amicon XM 50,000 membrane), and sterilised by Millipore filtration.

121 (Culture filtrate) Strains A20 and A1257 from cases of mycotic abortion, V211 from caprine mastitis and D7 from dust, were cultured individually in Roux bottles under the same conditions as 112. Equal volumes of the culture filtrates were mixed, dialysed, concentrated x 10 with Carbowax and sterilised by Millipore filtration.

170 (Culture filtrate) Strains A20, A1257, V211 and V215 were

used; culture and preparation as for 121.

138 - 142 inclusive (Culture filtrate) The antigens were prepared individually from the following strains isolated from cases of mycotic abortion: A 1815, A1847, A1848, A1849, A1844. These were cultured on 4% glucose 3% peptone for 4 weeks at 28°C, thereafter preparation as 121.

138M - 142M (Mycelial extract) The mycelium from each of the above strains was extracted with Coca's saline for 10 days at 4°C, dialysed and concentrated x 10 with Carbowax. Sterilisation by Millipore filtration.

154 (Mycelial extract) Strains A1923 from a case of mycotic abortion and V321 from aspergillosis in a budgerigar were cultured in Czapek-Dox (Oxoid) liquid medium in a fermentor, agitated by the passage of sterile air, at 28°C for 4 weeks. The mycelium was collected by centrifugation at 7,000 r.p.m. for 15 min., extracted with Coca's saline, dialysed, concentrated x 4 with Carbowax and sterilised by Millipore filtration.

193 (Culture filtrate) A20 was cultured in unshaken culture on Yeast malt (Difco) liquid medium at 28°C for 7 days. Preparation then as 121.

194 (Culture filtrate) A20 on 4% glucose 3% peptone liquid medium at 28°C for 7 days. Preparation as for 193.

196 (Mycelial extract) The mycelium from 194 was treated as 121 but concentrated x 10 with Carbowax.

197 (Mycelial, mechanical disruption) The mycelium from 193 was ground with sterilised silver sand in a mortar until homogenised and was then disrupted in a Mickle shaker with grade 12 Ballotini beads. Disruption was continued until microscopic examination of samples mounted in Cotton blue in lactophenol showed that most of the cells had been broken. The sand and beads were removed by centrifugation at 20,000 r.p.m. for 45 minutes. The supernatant was removed and sterilised by Millipore filtration.

195 (Complete antigen) A20 was cultured on Czapek-Dox (Oxoid) agar in Roux bottles at 28°C for 6 weeks. The agar and mycelium were then frozen and thawed 3 times, the extract was collected, dialysed for 48 hours against running tap water then sterilised by Millipore filtration.

M1 (Composite antigen) 1ml of each of antigens 193, 194, 195, 196 and 197 were mixed.

A. flavus (Culture filtrate) The fungus was cultured for 6 weeks at 28°C on 4% glucose 3% peptone. Preparation thereafter as 121.

A. niger (Culture filtrate) Preparation as for A. flavus.

A. ochraceus (Culture filtrate) Preparation as for A. flavus.

A. terreus (Culture filtrate) Preparation as for A. flavus.

Pityrosporum pachydermatis (Culture filtrate, Yeast cell and Composite) Strains 5851 and 5876 were cultured individually on Y.M. and 4% Malt extract for 7 days at 28°C. The yeast cells were then separated from the culture medium by filtration. The filtrate was dialysed for 24 hours against running tap water, concentrated x 10 with Carbowax and sterilised by Millipore filtration. Yeast cell antigens were made by disruption in

a Mickle shaker until microscopic examination of samples mounted in Cotton blue in lactophenol showed that most of the cells had been broken. The beads were removed by centrifugation at 20,000 r.p.m. for 20 minutes and the supernatant was sterilised by Millipore filtration. The composite antigen was prepared by mixing 1ml of each of the 8 antigens.

Antisera

Antisera to A. fumigatus, A. flavus, A. niger, A. ochraceus, A. terreus and P. pachydermatis were raised in adult New Zealand White rabbits. 0.5ml of a mixture of equal volumes of antigen and Freund's Complete Adjuvant were inoculated into the thigh muscle. A booster dose of 0.3ml antigen was injected into the marginal vein of the ear 21 days later. Serum samples were taken after 2 weeks, then at weekly intervals until the sera showed well-defined precipitation lines when the rabbits were given an overdose of anaesthetic and bled out by cardiac puncture.

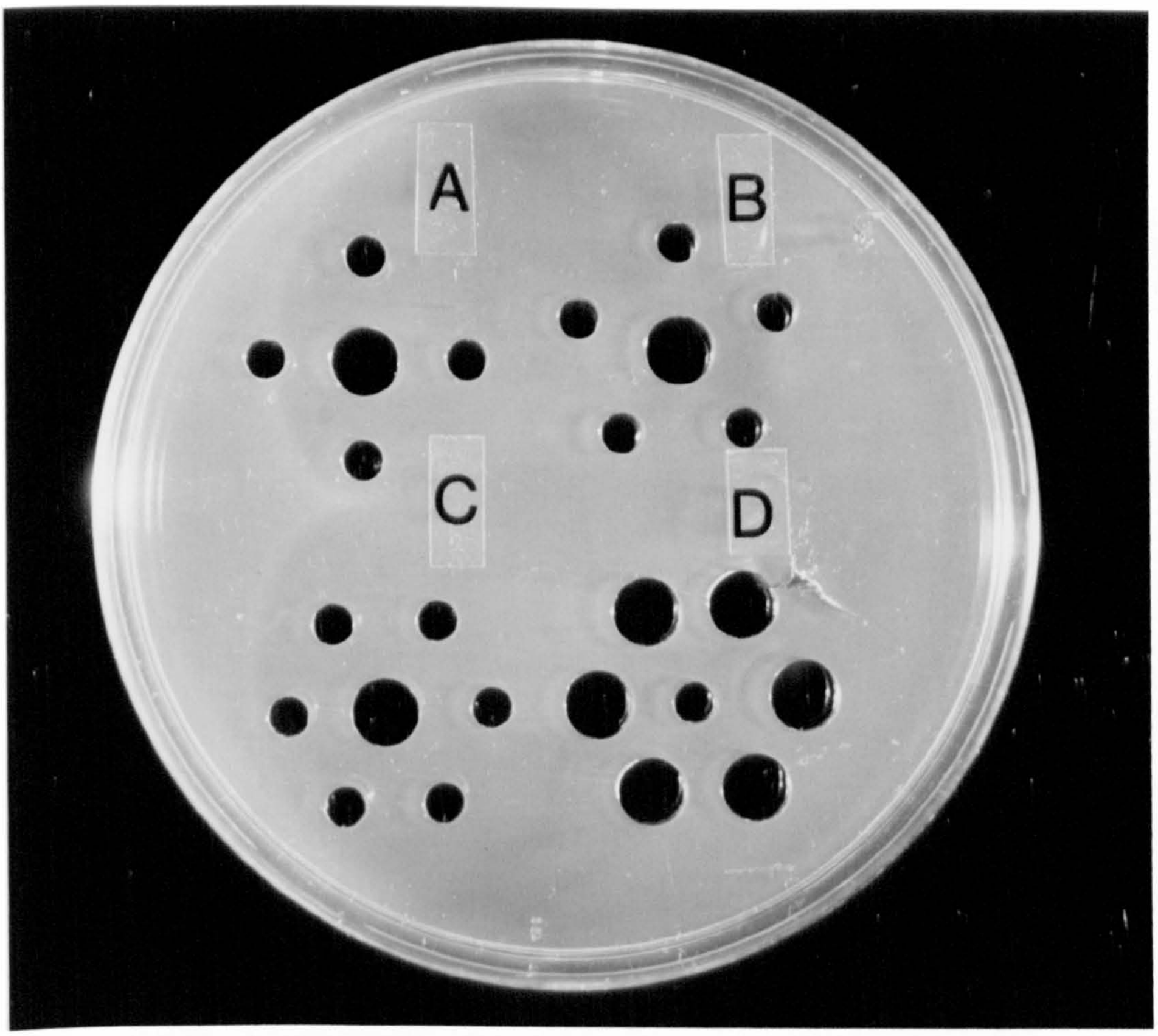


Fig. 1. The patterns used in Double Diffusion tests.

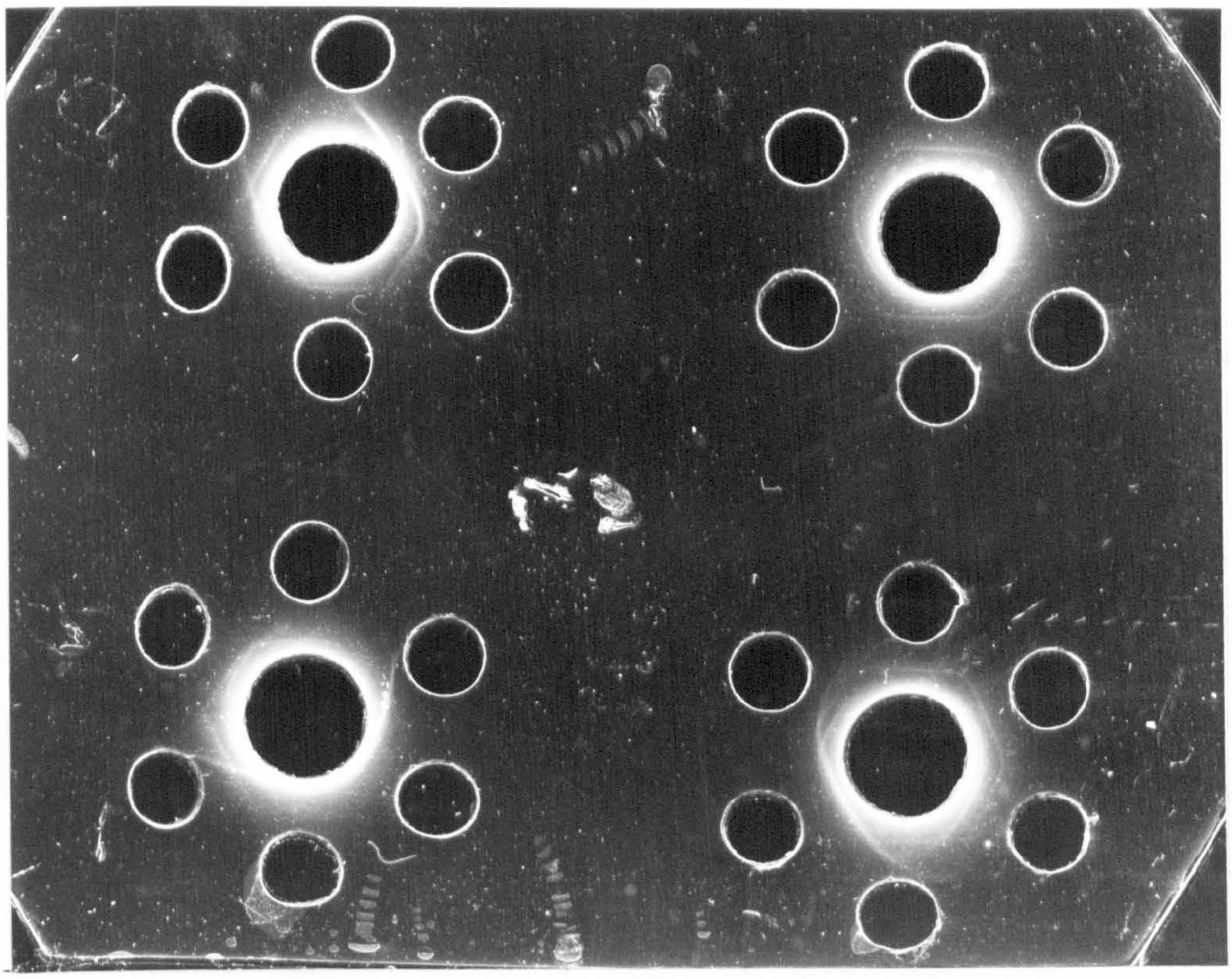


Fig. 2. Double Diffusion test: lines of precipitation after staining with Amido Black(negative print).

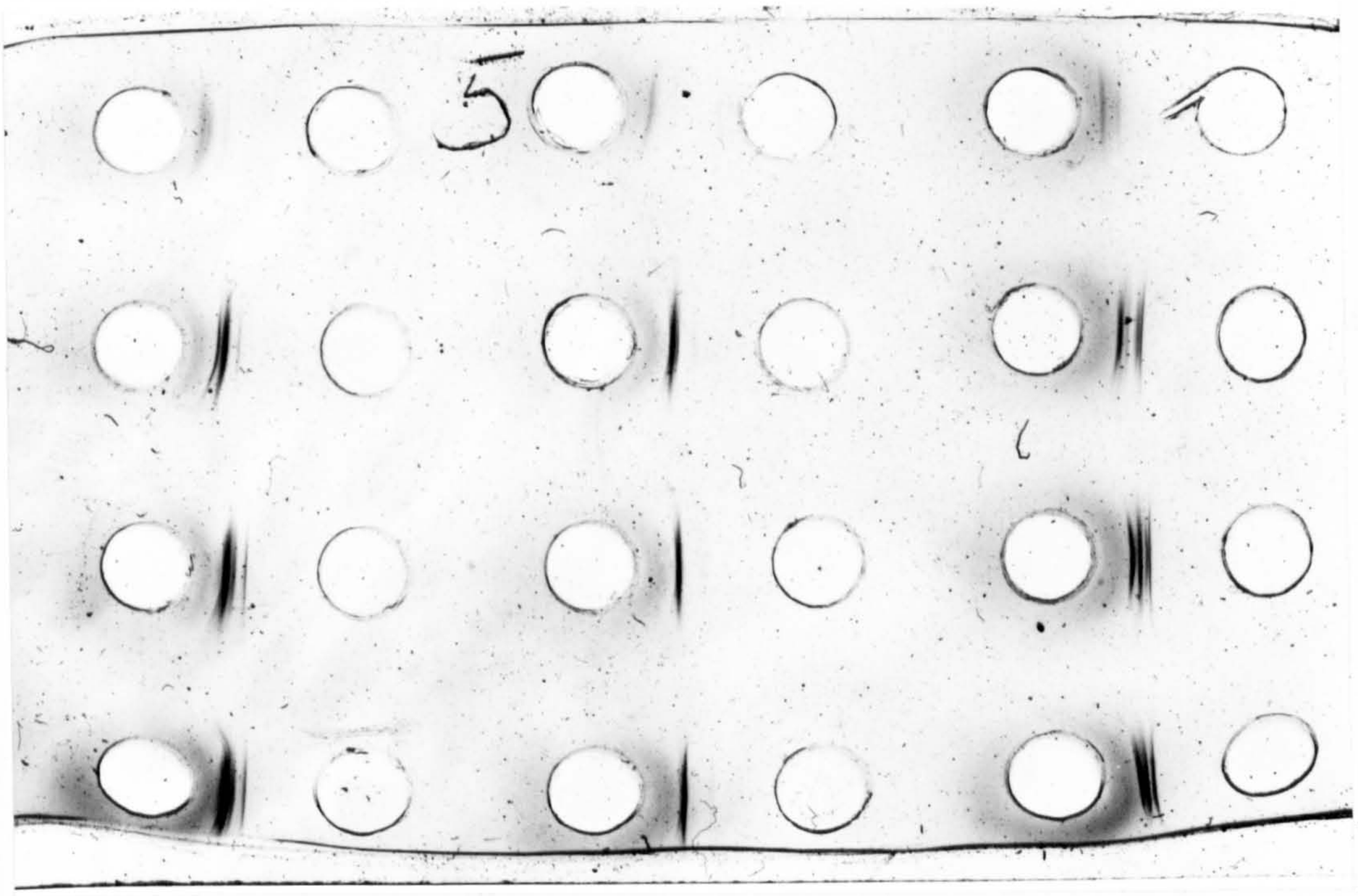


Fig. 3. Counterimmunoelectrophoresis test: lines of precipitation after staining with Amido Black.

COMPARISON OF ANTIGENS PREPARED FROM FIVE STRAINS OF *A. FUMIGATUS* CULTURED UNDER DIFFERENT CONDITIONS.

INTRODUCTION

Sartory in 1922, according to Matsumoto (1929) first reported on the serology of *Aspergillus*, with the first attempt to distinguish *A. fumigatoides* (*A. fischeri*) from *A. fumigatus*. Matsumoto (1929) used agglutination, precipitation and complement fixation tests with antisera to the different species of *Aspergillus* raised in rabbits as a method of species differentiation. None of his tests gave reliable results. Another approach to the use of the immune response was that of Bøe, Hartman and Thjøtta (1939) who tried to find if the antibodies produced in rabbits inoculated with different strains of *A. fumigatus* were related. The results were disappointing and they concluded that "Demonstration of antibodies in patients suffering pneumomycosis will hardly be successful".

Henrici (1939) described the suitability of *A. fumigatus* for experimental studies of fungal infections due to its constant virulence for laboratory animals and its little practical importance in man and animals. Although his work was mainly concerned with the endotoxins of *A. fumigatus*, he used the fungal cell sap as an antigen and raised antisera in rabbits.

The opinion that aspergillosis was of little importance as a disease of man and animals has changed due to the development of better methods of diagnosis, in which serological tests play an important part.

The most commonly used test in diagnosis of aspergillosis is the agar gel double diffusion test. This test was first used with A. fumigatus antigens as a diagnostic method by Pepys et al (1959). Their report triggered off a series of studies on the serology of aspergillosis which clarified allergic and infective aspects of this disease (Pepys, 1969).

Longbottom and Pepys (1964) described two different antigenic fractions in A. fumigatus antigens. One of the fractions was protein and the other polysaccharide. They also found substance C in some antigens prepared from cultures more than 5 weeks old which could react with the serum of a suspected case and give a false positive. Biguet et al (1962) used hyperimmune rabbit sera in the immuno-electrophoresis test to show that A. fumigatus could produce between 15 to 25 water soluble antigenic fractions. In 1964, Biguet et al, using the same test, found that culture filtrate and mycelial antigens were practically identical if obtained after one month's culture, except that there was one fraction missing from the culture filtrate antigen.

Tran van Ky, Biguet and Fruit (1966) studied serum samples from patients with A. fumigatus aspergilloma by I.M.E. and named four arcs, C, F, J and M which could be of importance in diagnosis, especially C and J which were nearly always present. Biguet, Tran van Ky, Fruit and Andrieu (1967) found that C had chymotryptic

activity and Tran van Ky, Biguet and Vaucelle (1963) described catalase activity in arc J.

English and Henderson (1967) compared the results of microscopy of sputum from cases of aspergillosis with those of culture and of demonstration of serum precipitins. They found that direct microscopy had diagnostic value but was time-consuming: they agreed with Longbottom and Pepys (1964) that a battery of antigens was needed to cover the range of precipitins produced against A. fumigatus.

In 1969, de Vries and Cormane studied 119 different strains of A. fumigatus and one of A. fischeri to determine if they varied in morphological, physiological and biochemical characteristics when isolated from patients and from different substrates. They found that no constant differences existed between strains but that strains isolated from patients were markedly different from the wild type.

Using neopeptone dialysate medium, Thurston, Richard and McMillen (1973) compared antigens prepared from 9 strains of A. fumigatus. They obtained the best antigens after 6 week's incubation at 27°C. Burrel and Thomas (1977) suggested that the carbon source in standard Sabouraud's broth should be reduced from 4% glucose to 1% to improve antigen production and to reduce variability of the antigens. Kurup, Fink, Scribner and Falk (1977) used AOAC medium and Czapek-Dox with 1% glucose as culture media and compared the antigens from 11 strains of A. fumigatus by D.D.. Their results showed that the reactivity

of the antigens against known positive sera varied from 42 - 87% positive among the different strains. In 1980, these authors tested 1310 sera with antigens prepared from 3 strains and concluded that antigenic preparations from one strain of A. fumigatus is not sufficient to detect antibody in the sera of all patients. On the other hand, Kim, Chaparas, Brown and Anderson (1978) who used culture filtrate and mechanically disrupted mycelial mat extract antigens prepared from A. fumigatus grown on synthetic medium found that the protein content of the extract from young mycelium was higher than that of young culture filtrate antigens. This phenomenon was inverted with age, suggesting that denaturation of the mycelium had occurred. They also found that antigens extracted from the mycelium were more sensitive than those commercially available.

Atkinson and Memon (1977) used a Sephadex column to purify antigens and suggested that precipitating antigens were protein only and not polysaccharide as suggested by Longbottom et al (1964), who found that after precipitating the proteins with Ammonium sulphate, the polysaccharide fraction, recovered from the supernatant fluid, produced lines in D.D. tests.

Kauffman and de Vries (1980) studied the antigenic composition of A. fumigatus culture filtrates. They found that the growth of the fungus caused changes in the pH of the medium which, in turn, depended on the concentration of glucose in the medium; high concentrations of

glucose inhibited autolysis of the fungus and the liberation of antigenic components.

In 1978, Reed noticed that A. fumigatus was variable in its growth. Flasks containing Czapek-Dox medium from the same batch were inoculated with a standard number of spores from the same culture and were incubated under the same conditions. The cultures varied considerably from flask to flask in rate of growth, pigmentation and sporulation. Such variations increase the difficulty of obtaining standard antigens and raising standard antisera in rabbits. This became more evident as more sensitive tests were developed.

Hipp et al (1970) described a latex slide agglutination test for the detection of antibodies against Aspergillus spp.. They suggested that this test could be used as a rapid initial screening test for large numbers of sera. Although there were some false positive results with this test the authors suggested that the problem could be solved by further testing.

Flaherty, Barboriak, Emanuel,, Fink, Marx, Moore, Reed and Roberts (1974) carried out an investigation on the reproducibility of 3 different methods of detecting precipitins against A. fumigatus. The tests were done in 3 different laboratories, using the same antigens and the same sera. The tests which were compared were micro D.D., Wadsworth template technique and C.I.E.. As they found that the first 2 methods were superior to C.I.E. in reproducibility, they

recommended the use of D.D. in routine analysis. In 1975, Dee investigated 9 cases of aspergillosis by D.D. and C.I.E. and found that C.I.E. compared favourably with D.D. for the detection of A. fumigatus antibody and had the added advantage that the time required to detect precipitins was reduced. These diagnostic methods were also compared by MacKenzie and Philpott (1975) who found that although C.I.E. required some experience in recognising positive tests it had two advantages over D.D., viz, greater speed and greater sensitivity. They also noted that mycelial antigens reacted in C.I.E. more frequently than in D.D. and pointed out that C.I.E. could be used instead of concentration of sera with low levels of antibody and re-testing them.

Other factors are involved in the serology of aspergillosis; one, apart from those already mentioned, is the antibody response of the patient to the disease. This has been noted by Longbottom, Augustin and Hayward (1960); patients with aspergilloma showed strong multiple lines in D.D. but those with allergic bronchopulmonary aspergillosis gave rather weak reactions. Bardana, McClatchy, Farr and Minden (1972) studied the type of antibodies present in sera from normal persons compared with patients with aspergillosis and confirmed the findings of Longbottom et al (1960) that in those patients with aspergilloma the majority of the precipitating antibody was IgG. IgM was also present but no correlation between them was demonstrated

in allergic bronchopulmonary aspergillosis in which the authors found IgE antibody to Aspergillus was incresed and IgG was decreased or lacking.

Scholer (1977) described a difference among antigens obtained from mycelium and those from mechanically disrupted spores. Since allergic aspergillosis is more frequently related to spore inhalation, this difference could be of importance in the diagnosis of that particular type of aspergillosis.

Specific precipitins could not be demonstrated in 9 of 15 immunosuppressed patients suffering from widespread invasive aspergillosis studied by Young and Bennet (1971). In 1978, Lehmann and Reiss detected, by C.I.E., circulating antigen in invasive aspergillosis in man and rabbits. In 1979 they characterised the antigen in the serum as galactomannan and they suggested that C.I.E. be used to detect circulating antigen in suspected cases and in compromised patients.

Richardson, White and Warren (1979) used C.I.E. and enzyme linked immunosorbent assay (ELISA) for the detection of antigen and found that ELISA was more sensitive for detecting antigen in the serum from experimentally infected rabbits which had been immunosuppressed with cortisone.

In the present study, antigens were produced from strains of A. fumigatus isolated from cases of animal aspergillosis, using a variety of culture media and different temperatures of incubation.

These antigens were tested against sera from animals suffering from aspergillosis, from animals exposed to the fungus and from animals experimentally infected. As far as I am aware, this is the first time that a study of this type has been undertaken in the veterinary field.

MATERIALS AND METHODS

Strains of A. fumigatus used in the production of antigens were selected from stock cultures maintained on Czapek-Dox (Oxoid) agar slopes. Identification was confirmed using the criteria of Raper and Fennell (1965).

A20 - Isolated April 1966 from the stomach contents of an aborted bovine foetus.

A1944-Isolated June 1979 from a bovine placenta from a case of mycotic abortion.

V323 -Isolated April 1975 from a lesion of guttural pouch mycosis in a horse.

V526 -Isolated February 1979 from material obtained at surgery from the nasal cavity of a dog with nasal aspergillosis which had been treated with Thiabendazole. This strain was abnormal. The chains of spores were fewer and shorter than those of a normal strain.

V539 -Isolated May 1979 from a nasal swab from a dog with nasal aspergillosis.

Sera used to test the antigens -

S1 - From a cow with Farmer's lung. This cow was examined post mortem and showed no evidence of aspergillosis.

S2 - From a dog destroyed because of nasal aspergillosis.

S3 - From a cow inoculated I.V. with A. fumigatus spores.

Culture Methods used in the Production of Antigens.

The media and temperature at which the strains of A. fumigatus were cultured are given in Table 1. For each strain in each experiment, 12 bottles (Saniglas) 20 x 7 x 5cm containing 100ml of liquid medium were inoculated. These were incubated, unshaken, lying flat to give the maximum surface area of medium for fungal growth. Two bottles were harvested at weekly intervals from 1 to 6 weeks. The antigens were prepared individually from each bottle.

The spore suspension used to inoculate the bottles was made from an 8 day culture on C-D(Oxoid) agar, by adding 10ml of a 0.5% sterile solution of Tween 80 in deionised water and mixing with a Whirly-Mix. The spores in the suspension were counted, using a Hawksley chamber, and the suspension was then adjusted with sterile water to give an inoculum of 50×10^6 spores per bottle.

Culture Filtrate Antigens

After the period of incubation the medium was decanted and filtered through Whatman no. 1 filter paper into a sterile flask and the volume and pH measured. The filtrate was then placed in Visking dialysis tubing (8-32/32") and dialysed against running tap water for 24 hours. The antigens were then concentrated approximately x 10 with Carbowax (Polyethylene glycol M.W. 20,000) and sterilised by filtration through a $0.45^{m\mu}$ Millipore filter and stored at $-20^{\circ}C$.

Mycelial Antigens

After the medium had been drained from the bottles in which the strains had been cultured, 100ml of Coca's saline solution were poured into them and after replacing the cotton stopper, the bottle was shaken until the mycelial mat was thoroughly wetted and covered by the solution. After storage at $4^{o}C$ for 10 days the fluid was separated from the mycelium by filtering through Whatman no. 1 paper and was then transferred to dialysis tubing and dialysed against running tap water for 24 hours and concentrated approximately x 10 with Carbowax. After sterilisation by passing through a $0.45^{m\mu}$ Millipore membrane the antigen was stored at $-20^{o}C$.

Dry Weight of Mycelium (DWM)

After extraction with Coca's saline, the mycelium was placed on a pre-weighed plastic boat and dried for at least 5 days at $46^{o}C$, until a constant weight was obtained.

Standardisation of ntigens.

The culture filtrate and mycelial antigens were standardised, using the weight of the dried mycelium as a measurement for concentration or dilution. It was decided that 1g of dry mycelium should equal 10ml antigen. e.g. 0.97g DWM = 9.7ml antigen.

Determination of Protein Content

The protein content of the standardised antigens was measured by the method of Lowry, Rosebrough, Farr and Randall (1951).

Serological Tests

All antigens were tested by D.D. and C.I.E. against the 3 standard sera, using the methods already described.

Titration of Antigens

Doubling dilutions of test serum S3 were made with half-strength borate buffer up to 1/32. The serum dilutions were tested by D.D. using pattern D (Fig. 1) with the antigen in the central well. The plates were held in a humid chamber for 7 days, washed and stained with Amido black.

RESULTS

Dry Weight of Mycelium.

In Table 2, the dry weight of the mycelium is given for each antigen. For those groups which were cultured and harvested in duplicate the figure given is the average of the weights of the 2 mycelial mats. The $\bar{X}$ D.W.M. of each group of antigens is given and these have been graded from one to 10 in order of increasing weight.

The groups with the lightest $\bar{X}$ D.W.M., 0.34g, were 21 and 22 (strain V526) followed by 19 and 20 (strain A20) with 0.49g. These 4 groups were cultured on Yeast malt medium at 28°C. Next in order of increasing weight were groups 7 and 8, $\bar{X}$ 0.82g and 13 and 14, $\bar{X}$ 0.87g, from V526 cultured on G.P. medium at 28°C and 37°C respectively.

Groups 1 and 2, A20 on G.P. medium at 28°C produced a $\bar{X}$ of 0.97g while the same strain on the same medium at 37°C gave a $\bar{X}$ of 1.14g. Groups 5 and 6, V323 on G.P. at 28°C gave a $\bar{X}$ of 1.03g. Groups 17 and 18, strain V526, and 15 and 16, strain A20, both cultured on G.P. at 46°C gave $\bar{X}$s of 1.17g and 1.25g respectively. The heaviest yields of mycelium were found in groups 3 and 4, A1944, and 9 and 10, V539, both on G.P. at 28°C and both giving a $\bar{X}$ of 1.27g.

In groups 3 and 4, 9 and 10 and 15 and 16, it was noted that the D.W.M. of the 6th week was heavier than that of the 1st week while in all the other pairs the weight of the mycelium harvested in the 6th week was lighter than that of the 1st week.

Protein Content of Culture Filtrate and Mycelial Antigens, (Table 3).

Of the C.F. antigens group 21, V526 Y.M. at 28°C, had the highest protein content ($\bar{X}$ 8.25mg) and it was this group which had the lightest D.W.M. ($\bar{X}$ 0.34g). Group 19, A20 Y.M. at 28°C had $\bar{X}$ 6.8mg protein; this group also gave the highest protein content of an individual antigen, 13.61 mg/ml in the 4 week antigen. The highest protein content of the antigens prepared from strains cultured on G.P. was that in group 7, $\bar{X}$ 3.34, V526 cultured at 28°C. This was closely followed by group 1, A20, also on G.P. at 28°C with a $\bar{X}$ 3.32mg. Group 11, A20 on G.P. at 37°C yielded a $\bar{X}$ 3.31mg. Group 15, A20 G.P. at 46°C had the highest protein content at that temperature ($\bar{X}$ 3.19mg). Group 5 antigens, $\bar{X}$ 2.72mg, were produced by culture of V323 on G.P. at 28°C and group 13, V526 on the same medium but at 37°C gave $\bar{X}$ 2.27mg. Of the culture filtrate antigens, group 17 gave the lowest protein content, $\bar{X}$ 1.83mg, from V526 cultured on G.P. at 46°C.

The highest protein content found in a group of mycelial antigens was that of group 2 produced by culture of A20 on G.P. at 28°C, $\bar{X}$ 3.26mg. Groups 22 and 20, strains V526 and A20, cultured on Y.M. at 28°C gave $\bar{X}$s of 2.42mg and 2.28mg.

Six groups of mycelial antigens occupied the bottom part of the scale as far as protein content was concerned. Group 16, strain A20 on G.P. at 46°C had a $\bar{X}$ of 1.73mg and group 6 from V323 on G.P. at 28°C was $\bar{X}$ 1.71mg. These were followed by group 8, the mycelial counterpart of the best culture filtrate antigen, grown on G.P.

This group gave $\bar{X}$ 1.58mg protein/ml. Group 14 produced a $\bar{X}$ protein content of 1.45mg/ml. These antigens were from strain V526 grown on G.P. at 37°C. Last but one in the production of protein were the antigens of group 10 which were also the heaviest in D.W.M. and were obtained from strain V539 on G.P. at 28°C, with a protein content of $\bar{X}$ 1.34mg. The lowest group was 12, $\bar{X}$ 1.1mg/ml, in which the lowest antigen was found in week 2 with only 0.13mg/ml.

Three groups, mycelial antigens 4 and 18 and culture filtrate antigens 9 yielded the same protein content, $\bar{X}$ 2.23mg/ml. Group 4 were from A1944 grown on G.P. at 28°C, group 18 from V526 on G.P. at 46°C and group 9 from V539 on G.P. at 28°C. Group 3, A1944 on G.P. at 28°C yielded $\bar{X}$ 2.22mg/ml, only 1mg less than its mycelial counterpart (group 4).

It was noted that the protein content of the culture filtrate antigens increased with the age of the culture; higher protein contents were found in the majority of the groups in antigens from the 4th, 5th and 6th weeks. The pattern was less clear in the mycelial antigens. Groups 6, 8, 10, 12, 14, 20 and 22 showed a pattern of higher protein content in the young antigens of weeks 1, 2 and 3. The other groups, 2,4, 16 and 18 did not follow any particular pattern. Group 2 had a low (0.94mg/ml) protein content in week 1, sharply increased in the second (3.36mg/ml) then a slow steady decrease from the 3rd (3.27mg/ml) to the 5th (3.08mg/ml) week and a sharp increase in the 6th (5.78mg/ml). Group 4 started with 1.13mg/ml, increased in the second week to 3.16mg/ml

and then decreased steadily to 1.51mg/ml in the 6th week. Group 16 had in week one a protein content of 1.09mg/ml which decreased to 0.56mg/ml in week 2, rose to 1.39mg/ml in week 3, decreased to 1.13mg/ml in week 4 and increased to 2.50mg/ml in week 5 and to 3.76mg/ml in week 6. Group 18 showed a protein content of 2.43mg/ml in the first week, dropped to 0.52mg/ml in week 2, rose to 0.75mg/ml in week 3 and to 2.05mg/ml in week 4 and to 4.39mg/ml in week 5 then decreased to 3.29mg/ml in week 6.

Serological Investigation of Antigens.

The results of D.D. and C.I.E. tests for each antigen tested against S1, S2 and S3 are presented in Table 4 as are the number of precipitation lines formed in each test. The groups of antigens were graded on their ability to detect antibody to A. fumigatus in the 3 test sera by D.D. and C.I.E., (Table 5). In grade 1 was the only group in which the 6 antigens reacted with all 3 sera in both tests with a total of 36 positive results. This group (13) was cultured in G.P. at 37°C, strain V526. The type of antigen was culture filtrate. The mycelial antigens from group 8, V526 on G.P. at 28°C, gave 35 positive and only one negative, with the first week antigen tested against S1 in C.I.E. Group 14, the mycelial antigens of group 13, produced 34 positives, S1 being negative in the C.I.E. test with antigens of the 5th and 6th weeks.

Groups 21 and 22, V526 grown in Y.M. at 28°C, gave an equal number of 33 positive results. S1 was negative 3 times by C.I.E. with the antigens of group 21 weeks 1, 3 and 6 and the mycelial antigens of group

22, weeks 1, 2 and 4 were also negative with S1 in C.I.E. Groups 4 and 6, mycelial antigens of A1944 and V323 respectively, cultured in G.P. at 28°C gave 32 positives each. Both groups were negative with S1 with 1, 2, 3 and 6 week antigens.

Groups 2, 7 and 15 gave 31 positive results. Group 2, mycelial antigens of A20 grown in G.P. at 28°C failed to detect antibody in C.I.E. with S1 with antigens 1, 2, 3 and 5. Group 7, culture filtrate antigens from V526 on G.P. at 28°C week 1, failed to react with any serum in D.D. and with S1 and S2 in C.I.E. Group 15, culture filtrate antigens of A20 on G.P. at 46°C were negative with S1 4 times by C.I.E. with antigens from weeks 1, 2, 3 and 5 and by D.D. with antigen week 1.

Group 17, culture filtrate antigens of V526 on G.P. at 46°C weeks 1, 5 and 6 missed S1 by D.D. and weeks 2, 3 and 5 missed S1 in C.I.E. tests.

Group 19, culture filtrate antigens of A20 on Y.M. at 28°C, weeks 2, 3, 4 and 6 missed S1 by D.D. and antigens weeks 2 and 3 by C.I.E.

Group 1, culture filtrate antigens of A20 on G.P. at 28°C produced 29 positive results, missing S1 by D.D. and C.I.E. with antigens 1 and 2, S2 by D.D. with antigen week 1 and C.I.E. with week 2 and S3 by C.I.E. with week 2 antigen.

Group 5, culture filtrate antigens of V323 on G.P. at 28°C gave 28 positive results. Antigens from weeks 1 and 2 missed S1 by D.D. and C.I.E., S2 by D.D. antigen week 1 and C.I.E. by week 3 and S3 by C.I.E. in weeks 2 and 4.

Groups 9, 16 and 18 produced 26 positives. Group 9 were culture filtrate antigens from V539 grown on G. P. at 28°C. S1 was missed by antigens of the 1st, 2nd and 3rd weeks in both D.D. and C.I.E. and by the 5th week in C.I.E.; S2 was not detected by antigens of week 2 by D.D. and S3 was missed by antigens of the 2nd and 3rd weeks in C.I.E. Group 16, mycelial antigens of A20 on G.P. at 46°C, weeks 4, 5 and 6 failed to detect S1 by D.D.; antigen of week one failed to detect S2 in D.D. and those of the 1st, 2nd, 5th and 6th weeks missed S1 in C.I.E. and those of the 5th and 6th weeks missed S3 in C.I.E. Group 18, mycelial antigens of V526 on G.P. at 46°C, weeks 3, 4, 5 and 6 were negative with S1 by D.D. and 6 also failed to detect S2. S1 was missed by antigens of the 1st, 2nd, 5th and 6th weeks in C.I.E. as was S3 by antigen week 5.

Groups 3 and 20 were the least able to detect antibody in the 3 test sera, with only 25 positive results. Group 3 were culture filtrate antigens of A1944 on G.P. at 28°C. Antigens of weeks 1 to 5 missed S1 by D.D. as did antigen week 1 with S2. In C.I.E. tests antigens 1 and 2 gave negative results with S1, week 2 antigens also missed S3, week 3 missed S2 and week 4 missed S3. Group 20, mycelial antigens of A20 on Y.M. at 28°C, weeks 1, 3 and 4 failed to detect S1 by D.D. and no antigen detected antibody in this serum in C.I.E and those of weeks 4 and 5 missed S3 in the same test.

Of a total of 264 serological tests, 132 D.D. and 132 C.I.E.) performed with each of the test sera (Table 6), S1 gave 160 (60.6%)

positive results, 93 (70.5%) of D.D. tests were positive and 67 (50.8%) of C.I.E. tests were positive. S2 showed positive results in 253 (95.8%) tests, 125 (94.8%) by D.D. and 128 (96.9%) by C.I.E. S3 gave positive results in 250 (94.6%) of all tests and 131 (99.2%) of the 132 D.D. tests were positive and 119 (90.2%) of C.I.E.

Of the 93 positive results in D.D. with S1,42 (45.2%) were obtained with culture filtrate antigens and 51 (54.8%) with mycelial antigens. By C.I.E. 40 (59.7%) of the 67 positive results were obtained with culture filtrate antigens and 27 (40.3%) with mycelial antigens. Of the 125 positive results in D.D. obtained with S2, 61 (48.8%) were obtained with culture filtrate antigens and 64 (51.2%) with mycelial antigens. In C.I.E. 62 (48.4%) of the 128 positives were obtained with culture filtrate and 66 (51.6%) with mycelial antigens. S3 was positive by all but one antigen in D.D.,that which failed was the culture filtrate antigen from week 1, group 7. In C.I.E. 59 (49.6%) of the 119 positives were obtained with culture filtrate and 60 (50.4%) with mycelial antigens.

Details of the number of precipitation lines produced are given in Table6 . S1 gave a total of 194 lines, 111 (57.2%) in D.D. of which 47 were with culture filtrate antigens and 64 with mycelial antigens. In C.I.E. S1 produced 83 (42.8%) lines, 53 with culture filtrate and 30 with mycelial antigens. The highest number of lines produced by S1 in D.D. was 3 with antigen week 2, group 8, while in C.I.E. the highest number of lines was 3 detected only by antigen week 6, group 22. S2 produced the highest number of lines,493, 179 (36.3%) in D.D. and 314 (63.7%) in C.I.E. Of the 179 lines in D.D., 90 were with culture

filtrate and 89 with mycelial antigens. In C.I.E. 148 lines were formed with culture filtrate and 166 with mycelial antigens. The highest number of lines obtained with a single antigen with S2 in D.D. tests was 3 and 7 antigens gave this. In C.I.E. a maximum of 4 lines was given by 7 antigens. With S3 419 lines, 186 (44.4%) in D.D. and 233 (55.6%) in C.I.E., were observed. In D.D. tests 99 lines were with culture filtrate and 87 were with mycelial antigens and in C.I.E. 129 lines were produced by culture filtrate and 104 by mycelial antigens. The highest number of lines observed with an individual antigen in D.D. was 3 and 3 antigens showed this result; culture filtrate antigens from week 6, group 5, week 1 group 13 and week 2 group 21. In C.I.E. the highest number of lines with an individual antigen was 4 and 7 culture filtrate antigens showed that result.

The titres obtained by the different antigens varied from 0, i.e. a line of precipitation only with the undiluted serum, to 1/32 (Table 7). A total of 12 antigens, 10 culture filtrate and 2 mycelial, gave a titre of 1/32. Group 13 gave the highest titres, 3 antigens with 1/32 and 3 with 1/16. Of 21 antigens giving titre 1/16, 20 were culture filtrate. Titre 1/8 was given by 19 antigens, 11 culture filtrate and 8 mycelial. A titre of 1/4 was given by more than a third of the antigens, 46, of which 15 were culture filtrate and 31 mycelial. A titre of 1/2 was given by 20 antigens, 9 culture filtrate and 11 mycelial and 13 antigens gave titre 0. These antigens were all mycelial.

The pH of the culture filtrates, taken immediately after harvesting, is given in Table 8. In most cases the pH showed a steady increase in alkalinity with age. In groups 15 and 17, A20 and V526 cultured on G.P. at 46°C, the pH of the culture filtrate of the 1st week was higher than that of the 6th week. Groups 19 and 21, A20 and V526 on Y.M. at 28°C ahowed slightly higher pH values than the rest of the culture filtrates, especially in the 5th week with values of 9.0 for group 19 and 8.9 for group 21.

Table 1. The group numbers given to the 6 culture filtrate and the 6 mycelial antigens prepared from each strain under each condition of culture.

Strain	Medium	Temp.	Antigen group number C.F.	M
A20	G.P.	28°C	1	2
A1944	"	"	3	4
V323	"	"	5	6
V526	"	"	7	8
V539	"	"	9	10
A20	"	37°C	11	12
V526	"	"	13	14
A20	"	46°C	15	16
V526	"	"	17	18
A20	Y.M.	28°C	19	20
V526	"	"	21	22

Table 2. The dried weight of the mycelium (D.W.M.) produced by the 5 strains of A. fumigatus cultured under different conditions, is given for each week of culture and also the mean weight ($\bar{X}$) of the group on which the antigen groups have been graded from 1 to 10 in order of increasing weight.

Group number C.F.	M.	Dry weight of mycelium (g.) 1	2	3	4	5	6	$\bar{X}$	Grading on $\bar{X}$
1	2	1.10	1.16	0.92	0.94	0.83	0.92	0.97	5
3	4	1.31	1.42	1.25	1.28	1.14	1.24	1.27	10
5	6	1.26	1.14	0.94	0.94	0.98	0.96	1.03	6
7	8	0.89	0.80	0.90	0.85	0.75	0.77	0.82	3
9	10	1.27	1.34	1.30	1.20	1.26	1.29	1.27	10
11	12	1.45	1.33	1.19	1.02	0.98	0.92	1.14	7
13	14	1.17	1.02	0.88	0.80	0.66	0.72	0.87	4
15	16	1.36	1.33	1.20	1.16	1.23	1.27	1.25	9
17	18	0.85	1.25	1.16	1.16	1.28	1.36	1.17	8
19	20	0.55	0.61	0.51	0.43	0.43	0.45	0.49	2
21	22	0.41	0.40	0.32	0.28	0.33	0.31	0.34	1

Table 3. The protein content of 1 - 6 week antigens of A. fumigatus graded from 1 to 20 in decreasing order on the mean protein content.

	Group number	Protein content, mg/ml of antigens 1	2	3	4	5	6	$\bar{X}$	Grading on $\bar{X}$
Culture filtrate	1	0.75	1.80	2.32	4.85	7.50	2.75	3.32	4
	3	0.45	1.70	1.55	1.67	3.00	5.00	2.22	13
	5	1.25	2.00	4.50	3.15	3.60	1.85	2.72	8
	7	3.75	0.49	2.52	2.37	6.49	4.42	3.34	3
	9	1.50	1.05	2.00	3.00	2.65	3.20	2.23	12
	11	1.65	2.75	4.50	6.00	2.20	2.80	3.31	5
	13	2.00	2.35	3.35	1.50	2.35	2.10	2.27	11
	15	1.60	3.50	4.50	4.00	2.80	2.75	3.19	7
	17	0.85	1.93	2.66	2.10	1.75	1.70	1.83	14
	19	5.46	4.38	5.17	13.61	6.30	5.92	6.80	2
	21	5.51	10.42	6.14	6.77	10.05	10.61	8.25	1
Mycelial	2	0.94	3.36	3.27	3.13	3.08	5.78	3.26	6
	4	1.13	3.16	2.60	2.81	2.22	1.51	2.23	12
	6	3.74	2.73	2.06	0.24	0.82	0.68	1.71	16
	8	3.22	2.63	0.87	1.38	0.68	0.75	1.58	17
	10	3.40	2.02	1.30	0.77	0.18	0.37	1.34	19
	12	1.75	0.13	1.14	1.05	1.26	1.31	1.10	20
	14	1.87	0.15	0.59	2.55	2.20	1.39	1.45	18
	16	1.09	0.56	1.39	1.13	2.50	3.76	1.73	15
	18	2.43	0.52	0.75	2.05	4.39	3.29	2.23	12
	20	2.85	2.06	2.14	2.58	2.80	1.28	2.28	10
	22	3.36	2.93	2.29	2.41	2.41	1.16	2.42	9

Table 4. Details of the number of positive tests and of the number of lines produced when the 6 antigens in each group were tested against S1, S2 and S3 by D.D. and C.I.E.

Group	Serum	D.D. Antigens 1	2	3	4	5	6	D.D. Total + lines		C.I.E. Antigens 1	2	3	4	5	6	C.I.E. Total + lines	
1	S1	0	0	1	1	1	1	4	4	0	0	1	1	1	1	4	4
	S2	0	1	2	2	2	1	5	8	1	0	3	3	3	3	5	13
	S3	1	1	1	2	2	2	6	9	1	0	2	2	2	1	5	8
2	S1	2	1	1	2	1	0	5	7	0	0	0	1	0	2	2	3
	S2	2	2	3	2	1	1	6	11	3	1	2	3	3	4	6	16
	S3	2	1	1	1	1	1	6	7	2	2	2	2	1	1	6	10
3	S1	0	0	0	0	0	1	1	1	0	0	1	2	2	2	4	7
	S2	0	1	1	1	2	1	5	6	1	1	0	3	3	3	5	11
	S3	1	2	2	1	2	2	6	10	1	0	1	0	4	1	4	7
4	S1	1	2	1	1	1	1	6	7	0	0	0	1	1	0	2	2
	S2	1	1	1	2	1	1	6	7	3	2	3	2	3	3	6	16
	S3	1	2	1	1	1	1	6	7	2	3	2	1	1	1	6	10
5	S1	0	0	1	2	2	2	4	7	0	0	1	2	2	2	4	7
	S2	0	1	1	1	3	3	5	9	1	1	0	3	3	3	5	11
	S3	1	2	2	2	2	3	6	12	1	0	1	0	4	1	4	7
6	S1	1	2	1	1	1	1	6	7	0	0	0	1	1	0	2	2
	S2	1	1	1	2	1	1	6	7	3	2	3	2	3	3	6	16
	S3	1	2	1	1	1	1	6	7	2	3	2	1	1	1	6	10
7	S1	0	1	2	2	1	1	5	7	0	2	1	2	1	1	5	7
	S2	0	1	2	1	2	2	5	8	0	3	4	2	3	2	5	14
	S3	0	1	1	2	2	2	5	8	1	3	3	2	4	3	6	16
8	S1	1	3	1	1	1	1	6	8	0	1	1	1	1	1	5	5
	S2	1	1	1	1	1	1	6	6	1	1	1	2	2	3	6	10
	S3	2	1	1	1	1	1	6	7	1	2	2	2	2	2	6	11
9	S1	0	0	0	1	1	1	3	3	0	0	0	1	0	2	2	3
	S2	1	0	1	1	2	2	5	7	1	1	1	2	2	3	6	10
	S3	1	2	1	1	2	2	6	9	1	0	0	2	3	2	4	8
10	S1	2	0	2	1	2	1	5	8	0	0	1	0	0	0	1	1
	S2	2	1	2	1	1	1	6	8	2	2	2	3	3	4	6	16
	S3	2	2	2	1	1	1	6	9	2	1	3	1	2	1	6	10
11	S1	0	0	1	1	1	0	3	3	0	0	0	1	1	2	3	4
	S2	1	1	1	2	2	1	6	8	1	2	2	4	2	2	6	13
	S3	1	1	1	1	1	1	6	6	2	1	2	4	1	3	6	13
12	S1	1	1	1	0	0	0	3	3	1	0	1	1	1	0	4	4
	S2	2	2	1	1	1	1	6	8	3	3	4	2	3	2	6	17
	S3	2	1	1	1	1	1	6	7	2	2	2	1	1	0	5	8

Continued on next page.

Table 4 (Continued).

Group	Serum	D.D. Antigens 1	2	3	4	5	6	D.D. Total +	lines	C.I.E. Antigens 1	2	3	4	5	6	C.I.E. Total +	lines
13	S1	1	1	1	1	1	1	6	6	1	1	1	1	1	2	6	7
	S2	2	2	2	1	1	1	6	9	2	3	2	2	4	3	6	16
	S3	3	1	2	1	1	2	6	10	4	3	4	2	4	3	6	20
14	S1	1	1	1	1	1	1	6	6	1	1	1	1	0	0	4	4
	S2	1	1	1	1	2	1	6	7	3	3	3	2	3	2	6	16
	S3	2	1	1	1	1	1	6	7	2	3	2	2	1	1	6	11
15	S1	0	1	1	1	1	1	5	5	0	0	0	2	0	1	2	3
	S2	1	2	2	1	1	1	6	8	1	3	4	3	2	3	6	16
	S3	1	1	2	1	1	2	6	8	2	2	2	2	2	2	6	12
16	S1	1	1	1	0	0	0	3	3	0	0	1	1	0	0	2	2
	S2	0	1	1	1	1	1	5	5	3	2	3	2	2	2	6	14
	S3	1	2	2	1	1	1	6	8	2	2	1	1	0	0	4	6
17	S1	0	1	1	1	0	0	3	3	1	0	0	1	0	1	3	3
	S2	1	2	1	1	1	1	6	7	2	2	2	3	3	3	6	15
	S3	1	1	1	1	2	1	6	7	1	2	2	3	2	3	6	13
18	S1	1	1	0	0	0	0	2	2	0	0	1	1	0	0	2	2
	S2	1	1	2	1	1	0	5	6	3	2	3	2	2	1	6	13
	S3	2	1	1	1	2	1	6	8	3	2	2	1	0	1	5	9
19	S1	1	0	0	0	1	0	2	2	1	0	0	2	1	1	4	5
	S2	1	1	1	1	1	3	6	8	2	3	2	3	2	2	6	14
	S3	2	1	2	1	1	1	6	8	3	3	2	2	3	1	6	14
20	S1	0	1	0	0	1	1	3	3	0	0	0	0	0	0	0	0
	S2	1	2	2	2	2	1	6	10	2	3	3	3	2	3	6	16
	S3	2	2	1	2	1	1	6	9	2	2	3	0	0	1	4	8
21	S1	1	1	1	1	1	1	6	6	0	1	0	1	1	0	3	3
	S2	3	2	2	2	1	2	6	12	3	3	2	2	3	2	6	15
	S3	2	3	1	2	2	2	6	12	3	1	2	2	2	1	6	11
22	S1	1	1	2	2	2	2	6	10	0	0	1	0	1	3	3	5
	S2	3	2	2	2	3	2	6	14	2	3	3	3	2	3	6	16
	S3	2	1	2	2	2	2	6	11	2	2	2	2	1	2	6	11

Table 5. Gradings of the antigen groups according to the D.W.M., protein content and antigenic ability, D.W.M. & Protein, grades as in Tables 2 & 3; Antigenic ability, grades rise with efficiency.

Group number	$\bar{X}$ D.W.M.	$\bar{X}$ Protein	Antigenic ability					
			+ve tests			lines		
			D.D.	C.I.E.	both	D.D.	C.I.E.	both
1	5	4	3	5	8	7	10	9
3	10	13	6	6	11	11	10	15
5	6	8	3	6	9	3	10	6
7	3	3	3	3	6	5	2	3
9	10	12	4	7	10	9	13	13
11	7	5	3	4	7	11	6	11
13	4	11	1	1	1	4	1	1
15	9	7	2	5	6	7	5	7
17	8	14	3	4	7	11	5	10
19	2	2.	4	3	7	10	3	8
21	1	1	1	4	4	2	7	4
2	5	6	2	5	6	4	7	5
4	10	12	1	5	5	7	8	9
6	6	16	1	5	5	7	8	9
8	3	16	1	2	2	7	10	11
10	10	19	2	6	7	4	9	7
12	7	20	3	4	7	10	7	11
14	4	18	1	3	3	8	5	8
16	9	15	4	7	10	12	12	14
18	8	12	5	6	10	12	11	13
20	2	10	3	8	11	6	11	12
22	1	9	1	4	4	1	4	2

Table 6. The results given by the 3 test sera with 66 culture filtrate and 66 mycelial antigens in D.D. and C.I.E. tests.

Serum		Positive tests						Positive all tests
		D.D.			C.I.E.			
		C.F.	M.	both	C.F.	M.	both	
S1	Number	42	51	93	40	27	67	160
	%	63.6	77.3	70.5	60.6	40.9	50.8	60.6
	Lines	47	64	111	53	30	83	194
S2	Number	61	64	125	62	66	128	253
	%	92.4	96.9	94.8	93.9	100.0	96.9	95.8
	Lines	90	89	179	148	166	314	493
S3	Number	65	66	131	59	60	119	250
	%	98.5	100.0	99.2	89.3	90.9	90.2	94.6
	Lines	99	87	186	129	104	233	419

Table 7. Titres of the antigens against S3.

Group	Weeks 1	2	3	4	5	6
1	1/2	1/2	1/4	1/8	1/8	1/8
3	1/4	1/2	1/4	1/4	1/4	1/4
5	1/32	1/4	1/16	1/16	1/8	1/16
7	0*	1/4	1/4	1/4	1/4	1/4
9	1/4	1/4	1/2	1/2	1/2	1/2
11	1/2	1/4	1/16	1/16	1/8	1/16
13	1/32	1/16	1/32	1/16	1/16	1/32
15	1/2	1/16	1/32	1/16	1/8	1/8
17	1/8	1/16	1/32	1/16	1/16	1/16
19	1/8	1/8	1/16	1/32	1/32	1/16
21	1/16	1/16	1/8	1/32	1/16	1/32
2	1/2	1/4	1/4	1/4	1/2	0
4	1/2	1/4	1/4	1/4	1/8	1/32
6	1/8	1/8	1/8	0	0	0
8	1/4	1/4	1/4	1/4	1/4	1/4
10	1/4	1/4	1/4	1/2	1/4	0
12	1/4	1/2	0	1/4	1/2	1/2
14	1/4	1/4	1/4	1/2	0	0
16	1/8	1/2	1/2	1/4	1/4	1/4
18	1/4	1/2	0	0	0	0
20	1/16	1/4	1/4	1/4	1/8	0
22	1/32	1/4	1/8	1/4	1/8	1/4

* 0 = +ve only with undiluted serum

Table 8. pH values of culture filtrates from week 1 to 6.

Group	Weeks 1	2	3	4	5	6
1	8.0	8.2	8.2	8.6	8.6	8.8
3	8.0	8.2	8.4	8.4	8.7	8.7
5	8.0	8.2	8.4	8.4	8.5	8.7
7	8.0	8.2	8.5	8.5	8.6	8.6
9	7.2	7.4	7.6	7.7	8.1	8.3
11	7.8	8.2	8.6	8.6	8.6	8.5
13	8.2	8.6	8.6	8.3	8.4	8.3
15	8.0	8.3	8.1	8.2	8.2	7.8
17	8.1	8.5	8.1	8.2	8.1	8.0
19	8.0	8.7	8.7	8.8	9.0	8.8
21	8.0	8.9	8.8	8.8	8.9	8.7

DISCUSSION

Strains of A. fumigatus can vary considerably in morphology and Raper and Fennell (1965) stated that strains isolated from human and animal tissues may have a different morphology from those isolated from natural substrates. This was also noted by de Vries and Cormane (1969) who studied 119 strains of A. fumigatus isolated from natural sources and from pathological specimens.

The statement made by Reed (1978) summarises the problem of the variability of A. fumigatus in morphology and as a producer of antigens: "The organism is extremely variable in its growth. Even on a defined medium, it is difficult to prepare two identical batches of antigen." The variation in ability of strains to produce antigens has been demonstrated by several workers (Thurston et al, 1973; Kurup et al, 1977; Bardana, 1978), and was also evident in the present studies.

In laboratories involved in work with A. fumigatus and particularly in those using antigens as diagnostic tools, it is common practice to have a number of "pet" strains which experience has shown to be good producers of antigen. In most cases, however, the selection of strains which are good producers of antigen is a matter of trial and error.

In this study, 5 strains of A. fumigatus were used, of which 4 were normal in morphology, A20 and A1944 from cases of mycotic abortion, V539 from a case of canine nasal aspergillosis and V323 from a case of

Guttural pouch mycosis. The 5th strain, isolated from canine nasal aspergillosis, was selected because of abnormal morphology. Parts of the colony were white and floccose and, although the rate of sporulation was good, the chains of spores were much shorter than those of a normal strain.

This abnormal strain produced the best antigens but the group of antigens produced by the other strain from nasal aspergillosis was 4th in antigenic ability. Of the strains from mycotic abortion cases, A20 antigens were 2nd best and those of A1944 were the least effective. The one strain from a case of guttural pouch mycosis was 3rd.

It is well known that fungi can deteriorate in culture but it is not known if artificial culture over long periods of time will affect the ability of the fungus to produce good antigens. Strain A20, isolated in 1966, has remained unchanged morphologically and still produces good antigens. Unfortunately as all the antigens produced from this strain shortly after it was isolated had been used up, it was not possible to compare them with those prepared for this investigation. It will be interesting to see if the abnormal strain V526 and the other normal strains can retain their efficiency as antigen producers. If these strains remain viable it would be worth reproducing the experiments in, say, 10 years.

The conditions under which the 5 strains of A. fumigatus were cultured affected the quality of the antigens which they produced.

The 3 groups of antigens with the best grading on antigenic ability in the serological tests were cultured on G.P. and the next 2 on Y.M. The temperature of incubation is also important. For the normal strains, incubation temperatures of 28°C or 37°C were equally satisfactory but V526 gave the best antigens at 37°C. Abnormally high temperatures of incubation such as 46°C appear to be detrimental to antigen production.

The duration of incubation also proved to be important in determining the quality of the antigens. Young, 1 - 2 week, culture filtrate antigens, from strains cultured on G.P. regardless of the temperature of incubation, were unable to detect antibody in S1, the "exposure" serum, by D.D. and C.I.E. Some mycelial antigens from young cultures also failed to detect antibody in S1.

Kauffman et al (1980) considered that the pH of the medium was an important marker for the antigenic composition and that cultures with high pH had a high amount of antigenic components. These authors found that the pH of Sabouraud's medium increased to 8.5 by the 2nd week and then either remained high or dropped slightly as the culture aged. In the present study, it was found that the general tendency of both culture media used was to increase sharply in pH in the 1st week and continue to increase gradually thereafter. In only 2 cases did the pH drop in the 6th week to a value lower than in the 1st week: A20 and V526 cultured at 46°C on G.P. It was also noted that these strains on Y.M. at 28°C gave slightly higher pH values than on G.P. at the same temperature and that the antigens were also better,

confirming the observation of Kauffman et al (1980).

There were differences in the weight of mycelium produced by the strains of A. fumigatus and this was also affected by the medium used and the temperature at which they were incubated. There was a very definite difference in the D.W.M. of A20 and especially of V526 on G.P. and Y.M. On the latter medium the D.W.M. of the strains was about half the weight of that produced on G.P. This probably reflected the preference of A. fumigatus for G.P. but the higher D.W.M. on this medium was not associated with better antigen production. Temperature of incubation affected the D.W.M. which, for strains A20 and V526 cultured at 28°C, 37°C and 46°C both showed an increase in D.W.M. with increasing temperature. Thus, if high yields of mycelium are required, a high temperature of incubation should be used.

From the $\bar{X}$ D.W.M. of the 5 strains cultured on G.P. at 28°C, it was apparent that V526 produced the least mycelium, that A20 and V323 were medium weight strains and that V539 and A1944 were the heaviest. It might be expected that when more mycelium was produced, the amount of antigenic material released from it into the culture fluid would be greater. This, however, was not so. V526 with the lightest D.W.M. was the best of the culture filtrate antigens, followed by the middle-weight strains A20 and V323. These results suggest that low D.W.M. may be due o rapid lysis and loss of metabolites into the culture medium.

Kauffman et al (1980) suggested that as the mycelium was lysed the protein content of the culture medium rose. Kurup et al (1980) noted that culture filtrate antigens had a higher protein content than mycelial antigens. The results of my study agreed with this with 2 exceptions. Strains A1944 on G.P. at 28°C and V526 on the same medium but at 46°C both showed higher protein content in mycelial antigens than was found in the culture filtrate antigens. The protein content of the antigens varied with the medium used. Those with the highest protein content were those grown on Y.M., V526 and A20, both of which produced low amounts of mycelium. This suggests a relationship between low D.W.M. and high protein content in the culture filtrate antigens.

Some of the antigens were found to have a very low protein content. Protein content is widely used to standardise antigens (MacKenzie and Philpott, 1975; Kaufman, 1976; Kauffman et al, 1980) and it is generally assumed that the higher the protein content, the better the antigen. From my results this was not the case. The group with the best antigenic ability was 13 which had a low protein content. On the other hand, group 21 with the highest protein content was rated 4th on antigenic ability.

The ability to demonstrate antibody in serum samples is the most important characteristic of an antigen. In this study D.D. and C.I.E. were used with 3 test sera to investigate the ability of the antigens, produced from various strains of A. fumigatus under various conditions of culture, to demonstrate the presence of antibody. Of the 3 sera used

S2, that from a case of canine nasal aspergillosis, gave a positive result with the most antigens (95.8%). With mycelial antigens, this serum was the only one which gave a positive reaction in all C.I.E. tests and was second in D.D. Cultura filtrate antigens also reacted well with this serum, being best in C.I.E. and second in D.D.

S3, from a cow inoculated I.V. with spores of A. fumigatus was that which did best in D.D. tests. It reacted with every mycelial antigen and with all but one on the culture filtrate antigens giving a total of 99.2% tests positive. In C.I.E. S3 reacted with 59 culture filtrate and 60 mycelial antigens giving overall 90.2% positive tests. S1, in which the antibody to A. fumigatus was believed to result from exposure rather than infection, was the least effective in D.D. and C.I.E. tests with the various antigens and particularly so with 1 and 2 week culture filtrate antigens prepared from G.P. cultures in D.D. tests. This could either mean that these early antigens were lacking some antigenic fraction or that antibody to these early antigens was not present in the serum. The use of these early culture filtrate antigens may therefore provide a means of differentiating antibody due to exposure from that due to infection. One result of exposure in man is allergy and Longbottom (1977) stated that in such cases the level of antibody is low. It would be interesting to examine a series of such cases with the young culture filtrate antigens to see if these would also fail to react.

Dick, Dawson and Campbell (1973) investigated the reactions of a

a series of antigens prepared from Micropolyspora faeni with human and bovine sera and found that the antigens which were best for one species were not those that were best for the other. In the tests with A. fumigatus antigens no marked difference in reaction was found in the sera from the infected dog and cow.in C.I.E. tests but in D.D. tests S3 was slightly better than S2.

According to Evans (1976) the number of precipitin lines produced by a serum in a gel diffusion test usually indicates the amount of antigenic stimulation to which the animal has been subjected. In the present study it was found that the number of lines produced depended not only on the amount of antibody present in the serum but also on the type of antigen used in the test and also on the test itself. Of a total of 1106 lines produced in all the tests, 630 (56.9%) were found by C.I.E. and 476 (43.1%) by D.D. This was to be expected as it is well known that C.I.E. is more sensitive in this respect as, under the influence of the electrical current the antigens migrate at different rates. In D.D. however, migration depends on the rate of diffusion of the antigens, a slower process and one which gives less good separation.

In D.D. tests S3 gave the highest number of lines (186) followed closely by S2 with 179 lines. These sera showed approximately equal numbers of lines with culture filtrate (99 and 90) and mycelial (87 and 89) antigens. S1 gave only 111 lines and also differed in its reaction to culture filtrate (47 lines) and mycelial (64 lines) antigens.

In C.I.E. tests, however, S2 showed the highest number of lines (314) and S3 gave 233. S1 was again the lowest with only 83 lines. With culture filtrate antigens S2 gave 148 lines and S3 129 lines and with mycelial antigens 166 and 104 lines respectively. S1, with culture filtrate antigens gave 53 lines and with mycelial antigens only 30.

Thus S1, the serum which gave the lowest number of positive tests (60.6%) was also that which showed the least number of lines in both D.D. and C.I.E. tests. S3 which was marginally better than S2 in the percentage of positive tests gave more lines in D.D. than S2 but in C.I.E. tests gave fewer lines.

The number of lines produced with a known positive serum in D.D. has been used as a method of antigen standardisation by Palmer, Kaufman, Kaplan and Cavallaro (1977). According to the results obtained in the present study, however, the number of lines which the various groups produced reflected their serological ability only to a certain extent. The best group of antigens (13) was rated fourth in D.D. but gave the highest number of lines in C.I.E. Group 22 which gave the best results in D.D. was rated fourth in the C.I.E. tests.

The type of antigen also affected the results with regard to the number of lines which were produced. In D.D. tests culture filtrate antigens were best with S2 and S3 whereas for S1 mycelial antigens proved better. In C.I.E. tests, however, culture filtrate

were better than mycelial antigens in the number of lines revealed by S1 and S3 and the converse was the case with S2. These results show that, when testing antigens using the production of precipitation lines as an evaluation system, the type of serum to be used as well as the type of test should be taken into consideration.

It is generally accepted that high or rapidly rising antibody titre is indicative of infection. As far as I am aware, no comparative study of the titres obtained with a wide variety of antigens tested against the one serum has previously been reported. In this study, titres with the various antigens ranged from 0 to 1/32, the highest dilution tested. Culture filtrate antigens tended to show higher titres than did mycelial antigens.

High titres appear to be associated with good serological ability. Three antigens of Group 13 gave titres of 1/32 and three titres of 1/16. Groups 8 and 14, 2nd and 3rd overall, were mycelial antigens and therefore not directly comparable with those of group 13. However, the results do suggest that the type of antigen selected for titration tests is important.

As a result of these studies it has become apparent that, from a practical point of view, an antigen is only as good as the antiserum used to test it and that it is important to select a suitable serum for use in antigen trials. Conversely, when one is preparing antigens for diagnostic work some thought should be given to the sera which are to be investigated and the test or tests to be used and the antigens prepared accordingly.

FUNGAL EXUDATES AS ANTIGENS.

INTRODUCTION

The presence of droplets of liquid on the surface of fungal mycelium was noted in 1887 by de Bary and it is now known that a number of species of fungi in each of the 4 main classes can produce exudates (Colotelo, 1978).

One species which has been intensively studied is Sclerotinia sclerotiorum. Cooke (1969) found that the exudate from the sclerotia contained carbohydrates. Jones (1970) showed that the exudate had phenol oxidase activity and contained proteins, amino acids and salts and Colotelo, Sumner and Voegelin (1971) that they contained proteins, peroxidases, lipids, amino acids and various enzymes.

Exudates have been used as a taxonomic feature in the genus Penicillium (Thom, 1930) and in the genus Aspergillus by Raper and Fennell (1965). These authors noted the presence of exudates in 10 of the 18 groups of the Aspergilli, including those containing potential pathogens such as A. fumigatus, A. terreus, A. flavus and A. niger.

McPhee and Colotelo (1977) who investigated the biochemical characteristics of Fusarium culmorum, suggested that the biochemical composition of the droplets as shown by electrophoresis, could aid in classification. No one has, however, suggested that as the exudates are apparently rich in fungal metabolites they might provide readily available antigens free from contamination by

constituents of the culture medium.

As various species of Aspergillus were producing exudate, it was decided to attempt to find the conditions giving the highest yields of exudates and to investigate the feasibility of their use as antigens in serological tests.

MATERIALS AND METHODS

The production of exudate was investigated in the following *Aspergillus* spp..

A. flavus. S4, isolated from a soil sample.

A. fumigatus. A20, isolated from a case of bovine mycotic abortion.

A. nidulans. 5905, isolated from an equine skin sample.

A. niger. E2, isolated from the shell of a turtle egg.

A. ochraceus. S9, isolated from a soil sample.

A. terreus. E4, isolated from the shell of a turtle egg.

Each species of *Aspergillus* was cultured on each of the following media.

Czapek-Dox (Oxoid) agar (C.D.A.) in Petri dishes and sloped in Universal bottles.

Czapek-Dox agar (Raper and Fennel, 1965) (C.D.A.R.) in Petri dishes and sloped in Universal bottles.

Czapek-Dox liquid medium (Oxoid) (C.D.L.) in 100ml amounts in 250ml flat bottles.

Yeast Nitrogen Base (Difco) agar (Y.N.B.) supplemented with glucose at 10, 20 and 30%, sloped in Universal bottles.

Y.N.B. supplemented with sucrose at 10, 20 and 30%, sloped in Universal bottles.

Y.N.B. supplemented with 10% mannitol, sloped in Universal bottles.

Two special culture techniques were used.

Cellophane technique. A block of agar, 3cm square, was removed aseptically from the centre of a plate of C.D.A., the well was then filled with sterile C.D.L. and a 4cm square of sterilised cellophane was placed over the well. The cellophane, over the liquid medium, was then inoculated.

Foam technique. A square (4 x 4 x 0.4cm) of foam plastic sterilised by autoclaving at 115°C for 10min.,was floated on the surface of C.D.L. in a Petri dish. The surface of the foam was then inoculated.

Each species was incubated at 28°C for 14 days and, with the exception of cellophane and foam cultures which were left undisturbed, was examined every day for the presence of exudate. This was harvested, using a capillary tube, on the 14th day. For each species, the exudates were pooled and sterilised by adding a drop of Merthiolate.

The protein content of exudates from A. fumigatus, A. flavus and A. niger was measured using the method of Lowry et al (1951). The volume of exudate from the other species was insufficient for protein estimation.

D.D. tests were carried out in Borate buffered agar, using well-pattern A. The exudate from each species was tested against antiserum raised in rabbits using conventional antigens and against serum samples with natural antibody.

RESULTS

The results of the experiments on exudate production are presented in Table 9.

A. terreus produced exudate in 12 of the 13 growth systems, A. ochraceus, A. nidulans and A. fumigatus in 6 and A. flavus in 5. A. niger failed to produce exudate. All 5 species produced exudate when cultured on Czapek-Dox (Oxoid) agar in Universal bottles, in liquid culture on Czapek-Dox (Oxoid) and in the agar well-cellophane technique. Exudate was obtained from 4 species in Petri dish culture on Czapek-Dox (Oxoid) agar: A. flavus was the species which failed. In Universal bottle culture on Czapek-Dox (Raper and Fennell) A. nidulans failed to produce exudate but the other 4 species did, and on yeast nitrogen base supplemented with 10% sucrose 4 species produced exudate but A. ochraceus did not.

Of the 3 carbohydrate sources tested, sucrose at 10% gave exudate in 4 species, glucose at 10% in 2 species and mannitol at 10% in one species. Higher amounts of sucrose and glucose in the medium led to reduced numbers of species producing exudate. Only 2 species produced exudate by the foam technique.

There was no marked correlation between the amount of growth made by the fungus and the amount of exudate produced. The time at which exudate production commenced ranged from 5 - 12 days after inoculation with most samples falling within the 5 - 7 day range.

For individual species, the time at which exudate production began varied with the different media and methods of culture. No correlation between the amount of growth of the fungus and the time of initiation of exudate was apparent.

The protein content of pooled exudates from A. fumigatus was 6.38mg/ml, that of A. flavus 4mg/ml and of A. nidulans 4.1mg/ml.

In D.D. tests exudate from each of the 5 species reacted with their relevant rabbit antiserum and produced sharp, clear lines; A. nidulans exudate gave 2 precipitation lines and the other species a single line. When A. nidulans extract was tested against 2 equine serum samples known to have antibody to this species, a positive result was obtained with one sample.

The exudate of A. fumigatus gave one line with S2 and 3 lines with S3 but failed to react with S1. A. fumigatus exudate was also tested using 12 serum samples from dogs positive for antibody to A. fumigatus: 9 sera proved positive, 4 giving 3 lines, 2 giving 2 lines and 3 giving one line. Details of these tests and a comparison with the results using other antigens are given in Table 10.

Table 9. Growth of and exudate production by species of Aspergillus on various media.

Culture medium	Aspergillus terreus			flavus			ochraceus			nidulans			fumigatus			niger			No.of spp giving exudate
	Gr.	Ex.	D.	Gr.	Ex.	D.	Gr.	Ex.	D.	Gr.	Ex.	D.	Gr.	Ex.	D.	Gr.	Ex.	D.	
Y.N.B. glucose 10%	3	2	12	3	-		2	-		3	1	5	3	-		3	-		2
Y.N.B. glucose 20%	3	2	12	2	-		3	-		2	-		3	-		3	-		1
Y.N.B. glucose 30%	3	2	12	1	-		1	-		2	-		1	-		2	-		1
Y.N.B. sucrose 10%	2	1	7	3	1	12	2	-		2	1	5	3	1	5	3	-		4
Y.N.B. sucrose 20%	3	-		2	-		3	1	7	3	-		3	-		3	-		1
Y.N.B. sucrose 30%	3	1	7	2	-		3	-		3	-		3	-		2	-		1
Y.N.B. mannitol10%	2	1	9	3	-		2	-		2	-		2	-		2	-		1
C.D.A.R. Universals	3	1	7	3	1	5	3	1	7	2	-		2	1	5	3	-		4
C.D.A. Universals	3	2	5	3	1	5	3	1	6	3	3	6	3	2	7	3	-		5
C.D.A. Petri dishes	3	1	8	3	-		3	2	9	3	3	6	3	1	6	3	-		4
C.D.A. Bottles	3	2	5	3	1	5	3	2	6	2	1	5	3	2	5	2	-		5
Cellophane technique	2	1		3	3		3	2		3	2		2	1		3	-		5
Foam technique	3	1		3	2		3	-		1	-		2	-		2	-		2
Total exudates		12			5			6			6			6			0		

Gr.= Growth 1 poor
2 good
3 excellent

Ex.= Exudate production 1 50+ λ
2 100+ λ
3 0.5+ml

D.=days until exudate produced.

Table 10.Comparison of the results of A. fumigatus exudate antigen with those of antigens 121, 170 and M1 tested by D.D. against 12 positive canine sera.

Serum	Titre	Number of lines given by			
		A.fumigatus exudate	121	170	M1
1	1/8	-	1	1	1
2	1/8	-	1	1	1
3	1/8	-	1	1	1
4	1/8	1	1	1	1
5	1/8	1	3	2	3
6	1/8	1	1	1	3
7	1/16	2	2	2	-
8	1/8	2	1	1	-
9	1/32	3	2	2	-
10	1/32	3	2	2	2
11	1/32	3	1	1	1
12	1/2	3	1	-	1

DISCUSSION

Of the 6 species of Aspergillus tested, only A. niger failed to produce exudate. Of those producing exudate, A. terreus was the most consistent, giving a positive result in 12 of the 13 tests, while the other species produced exudate in only about half of the tests.

Czapek-Dox (Oxoid) proved to be the best medium regardless of whether it was used in the form of solid or liquid medium; of the 2 special techniques, growth on cellophane gave much better production of exudate than on foam. It is possible that although the fungal species appeared to grow fairly well on the foam, it or some break-down product of it retarded or, in some cases, inhibited exudate production.

Of the 3 carbohydrates tested, sucrose at 10% gave the best results but these were less good than either of the Czapek-Dox media each of which contained 3% sucrose. It is therefore possible that lower amounts of carbohydrates in the Y.N.B. would give better production of exudate.

One physical factor which appears to be of importance in the production of exudate is high humidity. Exudates were produced more plentifully when the fungi were cultured in liquid medium, in Universal bottles and using the cellophane technique.

There w s no correlation between the amount of growth and the

time of initiation of, or amount of exudate. Colotelo et al,(1971) also observed this and suggested that exudate production was somehow related to active metabolism and that the formation of sclerotia or cleistothecia might be of importance.

The exudates contained proteins and, in the case of A. fumigatus, the amount compared favourably with some of the antigens from this species prepared and described earlier. That proteins were present in exudates was noted for other species of fungi by Colotelo (1973) who also showed the presence of a variety of enzymes and amino acids.

In my small series of tests, the exudates proved to be very good antigens. If there had been enough of the exudates to determine their optimum concentrations before use in the serological tests, the results might have been even better. However, if a strain does produce exudate it is, in my opinion, well worth culturing it in bulk and collecting the exudate for use as an antigen. Another advantage of exudates is that as they are produced on the surface of the colony they should be free from contamination by constituents of the culture medium.

In certain mycotic diseases of animals such as aspergillosis of the avian air sac, canine nasal aspergillosis and guttural pouch mycosis in horses, the fungus grows on the surface of the mucous membranes producing colonies comparable to those grown on nutrient agar in Petri dishes. As these sites are highly humid, it seems

possible that the fungus might produce exudates *in vivo*.

ANTIBODY TO *A. FUMIGATUS* IN SERA FROM NORMAL, EXPERIMENTAL AND ABORTING CATTLE.

INTRODUCTION

Without doubt, mycotic abortion is the most economically important fungal infection of cattle in Britain. Hugh-Jones and Austwick (1967) reported that 13 - 25% of all abortions were caused by fungi and similar figures were obtained for Scotland (Dawson, personal communication). Additional records on the incidence and other aspects of the disease are given by Campbell (1969) in his review of the subject.

The cow shows no sign of infection before abortion. The disease affects only the gravid uterus in which the main lesion is a mycotic placentitis. Cotyledons of an infected placenta retain much of their maternal portion and are very thickened, especially at the margins and the central area is often necrotic. The intercaruncular areas may be of a leathery consistency with discrete or confluent thickénings. Fungi may be present within the digestive tract of the foetus and in about 4% of cases skin lesions have been observed (Austwick and Venn, 1957).

The causal fungi are always present in the placenta of the affected animal and this is therefore the material of choice for diagnosis. Since the placenta is often retained, serological diagnosis would be of value as fungi are not always present in the aborted foetus.

Many species of fungi and yeasts have been recorded from cases of mycotic abortion but, without doubt, the fungus mainly associated with the disease is A. fumigatus which, according to Ainsworth and Austwick (1973) has been recorded from over 60% of cases.

There are few records of the presence of precipitating antibody to A. fumigatus in bovine sera. In Britain, Jenkins and Pepys (1965), as part of their investigations into precipitin reactions to mouldy hay, demonstrated antibody to this fungus in sera from normal cattle and from cattle with various types of respiratory disease. Angus, Gilmour and Dawson (1973) reported that 6% of sera from cattle from an abbatoir had antibody to A. fumigatus. Dolezalova, Dolezal and Pawlik (1973) and Pawlik (1975) also found antibody to be present in a proportion of the bovine sera which they investigated.

Corbel (1972) used an antigen prepared from the mycelium of A. fumigatus to test sera from normal cows and from cases of mycotic abortion. This author stated that precipitins were rarely encountered in sera from cattle with no history of abortion but that most sera from abortion cases in which Aspergillus sp. had been implicated contained precipitins to A. fumigatus mycelial antigens.

Sera from natural cases of mycotic abortion, from cases induced experimentally by inoculation of A. fumigatus and from abortions from causes other than fungal infection were investigated by D.D. and C.I.E.. In addition, sera from all adult cattle in 8 herds, 4 of which had an undiagnosed abortion problem were also studied.

MATERIALS AND METHODS

Serum samples, taken from the cow within 2 days of abortion, were submitted by veterinary practitioners. In 8 cases, 2 of A. fumigatus abortion and 6 diagnosed elsewhere, causal species not known, a second serum sample taken 14 days after the first was available. Sera from 8 cases of mycotic abortion, diagnosed only by microscopy, were provided by another laboratory. Sera from cows inoculated with spores of A. fumigatus to induce abortion were also examined as were sera from all adult animals in 8 herds, 4 of which had an undiagnosed abortion problem.

All D.D. tests were carried out in Borate buffered agar using well-pattern D. Abortion sera were tested using routine antigens 112, 121 and 170; sera negative with these were then tested with antigens 138 - 142 prepared from strains of A. fumigatus isolated from 5 of the cases of mycotic abortion. The sera from these 5 cases were also tested with mycelial antigens 138M - 142M using well-patterns D and A. The case from which A. terreus was isolated was also tested with an antigen prepared from this species. Herd sera and sera from experimental cattle were tested with antigens 112 and 121. A mycelial antigen, 154, was used in a series of tests with sera from natural and experimental cases of mycotic abortion and with sera from non-aborting cows.

In C.I.E. tests, antigens 121 and 170 were used as was an antigen (All) prepared from germinating spores in the following manner. Allantoic fluid from a cow in the 7th month of pregnancy and free from

mycotic placentitis was collected and immediately put into dialysis tubing which was then placed in a flask containing 6 times the volume of the allantoic fluid of sterile distilled water and left for 24 hours at 4°C. The dialysate was sterilised by filtration through a 0.45 mμ Millipore membrane and 100ml lots were transferred aseptically into 250ml sterile flasks. These were inoculated with a suspension of A. fumigatus (A20) spores, adjusted to give a final concentration of 10^6 spores/ml in the sterile water. The flasks were incubated at 37°C in a shaking water bath at 140 strokes/minute for 18 hours. After checking that the spores had germinated, the contents of the flask were filtered through Whatman no. 1 paper, dialysed for 24 hours against running tap water and sterilised by Millipore filtration.

I.M.E. was carried out in Veronal buffered agar, using antigen 121; sera from natural and experimental cases of mycotic abortion were tested.

Sera from aborting and normal cattle were titrated by D.D. against antigen 121.

RESULTS

Sera from 50 cases of naturally occurring abortion were investigated. Details of the cause of abortion and the results of the D.D. tests are presented in Table 11. Of the 23 cases of abortion caused by A. fumigatus 10 were positive with one or more of the routine antigens 112, 121 and 170. On further testing with antigens 138 - 142, 5 more cases were positive, giving a total of 15 (65.2%) cases positive. Paired serum samples from 2 cases of A. fumigatus abortion were investigated. The first sample from one cow reacted with one antigen and the second with 4. From the second cow, sample one was positive with 2 antigens and the second with 4. Six of the sera from A. fumigatus cases which reacted with 121 were titrated by D.D. Four sera gave titres of 1/2 and 2 sera titre 0. Titres from experimental abortion sera were 1/4 and 1/8. Arcs were not observed after I.M.E. using antigen 121 with any serum from a natural case of mycotic abortion but were produced by the sera from experimental cows.

Antibody was not demonstrated in serum from the case caused by an unidentified Aspergillus sp. nor in the serum from the case from which A. terreus was isolated. This serum also proved negative when tested with an antigen prepared from A. terreus.

Sera from 5 A. fumigatus cases negative with the routine antigens were tested against homologous culture filtrate antigens; 3 proved positive and 2 of these were also positive with their homologous mycelial antigen. Antibody could not be demonstrated in the remaining

2 sera even when these were concentrated X 2.

Antibody to A. fumigatus was found in 11 (44%) of the 25 cases of abortion not caused by an Aspergillus sp.

Mycelial antigen 154 was used in a series of D.D. tests with sera from natural and experimental cases of mycotic abortion and with sera from non-aborting cows. The results are presented in Table 12 from which it can be seen that the results from this antigen differed little from those which had been obtained by a culture filtrate antigen.

Details of the number of animals sampled and the number of sera with antibody to A. fumigatus in herds with and without an abortion problem are given in Table 13. The percentage of animals with antibody ranged from 0 to 24. Of the 239 sera from normal herds 15.5% had antibody whereas only 5.6% of the 324 sera from the herds with an abortion problem were positive.

The results of C.I.E. tests using antigens 121 and 170 are given in Table 14 in which brief details of the source of the serum and the results of prior D.D. tests are also given. Of the 18 sera from adult animals 14 were positive with antigen 121, 7 giving 1 line, 4 giving 2 lines and 3 giving 3 lines. With antigen 170, 11 sera were positive, 6 giving 1 line, 2 giving 2 lines and 3 giving 3 lines of precipitation.

The results of D.D. and C.I.E. tests in which the germinating spore antigen (A11) was used with 21 sera are given in Table 15. This antigen failed in D.D. but in C.I.E. 15 sera were positive, 1 with 2 lines and the remainder with 1 line.

Table 11. Details of the cases of abortion from which serum samples were obtained and the results of D.D. tests with routine antigens and, for sera negative with these, antigens 138 - 142.

Cause of abortion	Number of cases	sera	Positive with one or more of antigens 112,121,170 cases	sera	antigens 138 - 142 cases	sera	Total positive cases	sera
A.fumigatus	23	25	10	12	5	5	15(65.2%)	17
A.terreus	1	1	0	0	0	0	0	0
Aspergillus, not fumigatus	1	1	0	0	0	0	0	0
Mucoraceous moulds	6	6	3	3	0	0	3(50.0%)	3
Yeast	1	1	0	0	0	0	0	0
Micro +ve only	4	4	2	2	0	0	2(50.0%)	2
Fungal, sp. not known	9	15	4	7	1	1	5(55.6%)	8
Not fungal	5	5	1	1	0	0	1(20.0%)	1

Table 12. The results of D.D. tests using mycelial antigen 154 compared with those obtained with culture filtrate antigen 121.

Serum from	Number tested	Antigen 121 +ve	Antigen 121 -ve	+ve 121 +ve 154	+ve 121 -ve 154	-ve 121 +ve 154	-ve 121 -ve 154	Total +ve 154
A.fumigatus natural aboortions	9	4	5	3	1	2	3	5
A.fumigatus exptl. cattle	55	50	5	47	3	5	0	52
Normal cattle	68	63	5	55	8	4	1	59
Total	132	117	15	105	12	11	4	116

Table 13. Details of the number of cattle sampled and the number showing precipitating antibody to A. fumigatus in herds with an undiagnosed abortion problem and in normal herds.

Herd	Number of cattle	Number positive	% positive
1	25	0	0
2	85	20	23.5
3	75	4	5.3
4	54	13	24.1
5+	89	15	16.9
6+	50	0	0
7+	105	0	0
8+	80	3	3.7

+ Brucella-free herd with an undiagnosed abortion problem.

Table 14. The results of C.I.E. tests using antigens 121 and 170. D.D. results included for comparison.

Serum from	Number tested	D.D.		C.I.E.			
				121		170	
		+ve	-ve	+ve	-ve	+ve	-ve
A.fumigatus natural abortions	10	3	7	7	3	7	3
Mycotic abortion sp. unknown	3	0	3	2	1	0	3
A.fumigatus exptl. cattle	5	5	0	5	0	4	1
Total	18	8	10	14	4	11	7

Table 15. Results of C.I.E. tests using the germinating spore antigen (All) with sera from abortion cases and from normal and experimental cows.

Serum from	Number tested	Positive D.D. All.	Positive D.D. Routine	Positive C.I.E. All.	Positive C.I.E. Routine
A.fumigatus natural abortions	10	0	2	7	3
Mycotic abortion sp.unknown	3	0	0	2	0
A.fumigatus exptl. cattle	3	0	1	3	1
Normal cattle	5	0	0	3	2
Total	21	0	3	15	6

DISCUSSION

As a result of these studies it has become apparent that the presence of precipitating antibody to A. fumigatus in bovine serum is not a suitable method of diagnosing cases of mycotic abortion caused by this species. Serum samples from normal cattle and from cases of mycotic abortion caused by species other than A. fumigatus have been shown to have antibody and a proportion of sera from cases of mycotic abortion known to be caused by A. fumigatus did not.

These results do not agree with those of Corbel (1972). Because this author had used a mycelial antigen, it was decided to further test sera from cases of A. fumigatus abortion and from normal cattle with a mycelial antigen. The results of these tests, however, were much the same as those obtained with the culture filtrate antigens.

The antigen prepared from germinating spores of A. fumigatus (All) has the advantage of being a particularly pure antigen, as the substrate was sterile water with allantoic fluid dialysate which would be removed by dialysis after culture. This antigen failed in D.D. tests. This was not surprising as it was used unconcentrated but in C.I.E. it gave better results than the routine antigens, but still failed to demonstrate antibody in all serum samples from cases of A. fumigatus abortion.

The failure to detect antibody in sera from cases of mycotic abortion might be explained in a number of ways. The cow might have been a poor producer of precipitating antibody, or transfer of antibody

to colostrum after abortion might have depleted the maternal antibody. From the results obtained from experimental cows this explanation seems unlikely as antibody was demonstrable in the cows which were held for several weeks after abortion and in others which calved normally.

Another possible explanation is that the strain of A. fumigatus causing the abortion differed antigenically from the strain used to produce the antigens. However, 2 of 5 sera failed to react with their homologous culture filtrate and mycelial antigens, even when the serum had been concentrated.

A more likely explanation for the lack of detectable antibody in some of the cases is suggested by Thurston et al (1972) who investigated mycotic abortion experimentally in ewes. These were challenged by aerosol, by intravenous inoculation and by inoculation directly into the uterine artery. In those injected via the uterine artery abortion or placental infection resulted but the authors failed to detect antibody to A. fumigatus in the serum. They suggested that this might be due to the failure of uterine or placental tissues to act as a locus for the formation of antibody. If this were the case, the antibody detected in sera from aborting cattle would be that developed as a result of exposure to the fungus in their environment

ANTIBODY TO A. FUMIGATUS IN SERA FROM NORMAL CATS

INTRODUCTION

The first of the few cases of feline aspergillosis which have been recorded in the literature was that of Sautter et al (1955). These authors described a case in a young Siamese cat which had died after showing clinical signs of infection of the respiratory tract for 2 weeks. On histological examination of a lung section, hyphae typical of those of an Aspergillus sp. were found in a granulomatous lesion in the lung; 3 other cats had shown similar clinical signs but the other cat which died was not examined post mortem.

The case of pulmonary aspergillosis described by Pakes et al (1967), in a cat heavily infested with hookworm, was also diagnosed solely on histopathological evidence. A third case of pulmonary aspergillosis, recorded by McCausland in 1972, concerned a Siamese cat aged 2 which presented with anorexia and vomiting. It died 6 days later and necrotic areas in the lung were found to contain branching septate hyphae compatible with those of an Aspergillus sp..

Aspergillosis of the feline digestive tract has been reported and is generally regarded as a secondary disease, usually associated with feline infectious enteritis and the use of broad-spectrum antibiotics. Bolton and Brown (1972) described mycotic colitis in a 7 month old short-haired cat which had a history of anorexia and vomiting. At autopsy the abdominal cavity was found to contain a large amount of coffee-coloured fluid and the thoracic cavity was partially filled with a pale watery fluid. Plaques, 1 - 2cm in

diameter, were present in the wall of the colon which, on histolcgical examination, proved to be areas of haemorrhagic necrosis with numerous branching septate hyphae throughout them. Lethargia was the main clinical sign of disease in the case reported by Stokes (197?) in which the pathological findings were confined to the intestinal tract. The duodenum was ulcerated and haemorrhagic and petechial haemorrhages were seen in the jejunum and ileum. Septate hyphae were observed in the ulcerated lesions and identification of Aspergillus sp. was made on the morphological characteristics.

A case in which lung and ileum were infected by an Aspergillus sp. was described by Fox et al (1978) in an adult female short-haired cat with leucopenia which, in 10 days, changed to leukocytosis with a left shift. At autopsy, the thorax contained fluid; there were many pulmonary adhesions and numerous white spots were present on the surface of the lungs and pericardium. The abdominal cavity also contained fluid and there were multiple adhesions. Two types of intestinal lesions were seen, those compatible with feline panleucopenia and areas of acute diffuse necrosis extending through the musclature, sub-mucosa and mucosa. Aspergillus-type hyphae were seen in the lung lesions and in the necrotic areas of the gut. A second case described by these authors also occurred in a short-haired cat. This female was in very poor condition; it was very thin, infested with fleas and had a high temperature and pale mucous

membranes. An abscess was present on the 5th digit of the left hind foot. This was removed surgically and 2 days after the operation the cat was anorectic and had developed a thick, purulent, bilateral nasal discharge. Severe leucopenia was found and after 5 days leukocytosis with a left shift developed and the cat died. Hyphae of Aspergillus sp. were found in the lung lesions.

Wilkinson et al (1982) described orbital cellulitis and sinusitis in a 4 year old, long-haired male cat. Hyphae resembling those of an Aspergillus sp. were seen in the lesions but although a fungus was obtained in culture it was not further identified.

All 10 cases of feline aspergillosis described in the literature were diagnosed as such only at autopsy. This would suggest that cases which are not examined at autopsy are being overlooked and that there is a case for the use of a serological test for the presence of antibody to A. fumigatus, at least, as a means of diagnosing these infections in vivo. It was therefore decided to carry out a survey to determine if antibody to A. fumigatus was present in a normal cat population.

MATERIALS AND METHODS

A total of 120 sera from healthy cats were investigated by D.D. and C.I.E., using 8 antigens, 121, 170, 193, 194, 195, 196, 197 and M1.

RESULTS

Precipitating antibody to A. fumigatus was found in 5 (4.2%) of the 120 sera. The D.D. test gave a positive result with 2 sera; each of which gave a single precipitation line with antigens 121, 170, 193 and M1. Three sera were positive by C.I.E., each giving a single precipitation line with antigens 121, 170, 193 and M1. None of the 5 positive sera was positive in both tests.

DISCUSSION

This survey has shown that antibody to A. fumigatus is present in a small proportion of sera from healthy cats in the Glasgow district. About 200 cats are examined post mortem at the Veterinary School each year but, to date, no case of feline aspergillosis has been diagnosed. This parallels the finding of Nimir (1980): although antibody to Cryptococcus neoformans was present in 4.4% of 275 feline sera, no case of the disease had been diagnosed.

In comparison with the results from the 2 surveys on antibody to A. fumigatus in canine sera, the number of cats showing antibody (4.2%) was much lower than that from the dogs (14.6% in one survey and 31.4% in the other). As the amount of A. fumigatus in the environment would be the same, the variation may be due to anatomical differences or to differences in behaviour between cats and dogs.

That some sera were positive in the D.D. test and negative in C.I.E. and vice versa, cannot be easily explained. Negative in D.D. and positive in C.I.E. might be due to the greater sensitivity of the latter test enabling amounts of antibody too small to be detected by D.D. to be observed. No explanation for the converse result can be given.

It is to be regretted that in none of the cases of feline aspergillosis recorded in the literature, was any attempt made to identify the causal fungus beyond generic level, even in the one

case in which Aspergillus was obtained in culture. Although, in dogs, A. fumigatus is by far the commonest causal agent of fungal disease it is possible, although unlikely, that this species might not predominate in feline infections. If this were the case, A. fumigatus antigens might fail to demonstrate antibody to another species of Aspergillus unless this were present in high titre.

The survey has shown that it is possible to demonstrate antibody to A. fumigatus in feline sera and has given an idea of the proportion of apparently normal animals liable to have antibody. I would suggest that routine serological examination of cats with respiratory symptoms might enable a diagnosis of aspergillosis to be made in vivo.

STUDIES ON CANINE NASAL ASPERGILLOSIS

INTRODUCTION

The organ mainly attacked by Aspergillus sp. varies according to the species of animal. In the dog, the predilection sites for infection are the nasal turbinates and the frontal sinus and reports of canine aspergillosis involving other parts of the body are rare.

According to Hoare (1913), canine aspergillosis was first reported in 1871 by Gotti who described a mycosis of the external auditory canal and in 1885 by Rivolta who reported a case of systemic infection by a mycelial fungus which he named Muromyces canis familiaris.

A disseminated infection was reported by Isoun (1975) who described the case of a 5 year old Labrador which was in very poor condition, suffering from babesiosis and ancylostomiasis. At post mortem examination lesions were found in the heart, kidneys and pancreas and, in sections, septate branching hyphae morphologically resembling those of an Aspergillus sp. were found. Another case of disseminated aspergillosis was described by Wood, Hirsch, Selcer, Rinaldi and Boorman (1978) in a 2 year old dog. Treatment with amphotericin B-methyl ester failed and at autopsy lesions were observed in bone, myocardium, spleen, kidney, liver, lymph nodes and both eyes. This report is interesting because the causal agent was A. terreus, a species not previously described as pathogenic for the dog.

The only case of canine pulmonary aspergillosis recorded is that of Ohshima et al (1979) in a 2 year old female suffering from distemper.

She had been treated with high doses of antibacterial antibiotics for 15 days without remission of the symptoms then developed encephalitic syndrome and was destroyed. At autopsy, the lungs showed focal bronchitis and histopathological examination of the lesion revealed septate and Y-branching hyphae within the wall of the bronchus.

In 1982 Weitkamp described 2 cases of mycotic osteomyelitis caused by an Aspergillus sp.. The first case was a 2 year old German shepherd bitch with a lesion involving vertebrae C_4 and C_5, characterised by lysis and some bone production. The bone was softened and a wet preparation of this material revealed branching septate hyphae and on culture an Aspergillus, not further identified, was obtained. The second case was a 4 year old bitch of the same breed which had a lytic lesion of the pubis showing a "starburst" pattern of bone destruction and proliferation. A biopsy sample from the bone was almost fluid and contained branching septate hyphae. An Aspergillus sp. was obtained in culture.

These cases of aspergillosis in the dog are exceptional in that parts of the body other than the nasal turbinates and sinuses were involved. According to Hoare (1913) canine nasal aspergillosis was first described in 1905 by Stazzi in a 6 year old dog which, in addition to head-shaking and scratching and rubbing his muzzle, developed epileptiform fits. At post mortem examination, a lesion

on the inferior turbinate bone was described as a brownish patch covered with a layer of dark green mould. Microscopy showed this to be A. fumigatus and the identification was confirmed by culture. The pathogenicity of the isolate was demonstrated by intravenous inoculation into rabbits and pigeons. Stazzi's second case showed a muco-sanguineous discharge from the right nostril. A. fumigatus was obtained in culture from the discharge and from material removed from the frontal sinus when this was trephined.

After these early reports the condition did not occur, or more probably was not recognised again until 1966 when Malicka described a case in a 15 month old collie which was destroyed because of severe leptospirosis. At autopsy, A. fumigatus was isolated from a 9cm lesion in the nasal chamber. Otto (1970) who described a case of aspergillosis in the frontal sinus of a dog, probably set off the new spate of reports of cases of nasal aspergillosis by his warning that because of the belief that aspergillosis is primarily an avian problem, the possibility of its presence in the sinus of the dog was overlooked. In the following year Spreull (1971) described 2 cases, stated that antibody to A. fumigatus was present in the serum of infected animals and that this could be demonstrated by precipitation. In the same year Parker and Cunningham described successful surgical treatment of a case with clinical signs of involvement of the central nervous system. Soltys and Sumner-Smith (1971) treated, surgically

and with Amphotericin B, a dog which had a chronic nasal discharge and was suffering convulsions. Three months later it showed an alteration in temperament and impairment of its intellectual ability suggesting damage to the cerebral cortex. Unfortunately, although this dog was destroyed, post mortem examination was not permitted. A second case which was treated by surgery only, as the dog was suffering from chronic nephritis, also showed a recurrence of symptoms after about 3 months.

The first report of the isolation of a degenerate strain of A. fumigatus which produced vesicles with sterigmata but no conidia, was that of Weber and Rudolph (1972). The dog, a 4 year old collie, had had a muco-sanguineous discharge from the right nostril for 6 months and had been subjected to long-term and intensive treatment with cortisone and antibacterial antibiotics. From a second case described by these authors which had shown similar clinical signs for 4 months and which had been similarly treated, a normal strain of A. fumigatus was isolated.

Black and Mightingale (1973) who unsuccessfully treated a case by surgery and Amphotericin B in an Afghan hound which came from a farm and often slept in the stables suggested that there was the possibility of a relationship between infection and exposure to substrates heavily contaminated with A. fumigatus. Other factors predisposing to infection were suggested by Dawson, Baker and Mackey

(1973) who described concurrent nasal aspergillosis and tonsillar carcinoma in a 10 year old working collie which had been treated with antibiotics for 8 months. These were that the neoplastic disease might have predisposed to the fungal infection either by immunological impairment or by interference with the lymphatic drainage system.

No predisposing factors appear to have been involved in the case reported by Cadwallader, Goulden, Baxter, Wyburn and Alley in 1973, in an 8 year old working collie. Surgery was performed a month after the dog had convulsions and radiography showed an extension of the sinusitis into the cranial vaults. A. fumigatus was cultured from material removed from the frontal sinus. The dog was destroyed but no fungal elements were found in the brain.

In 1974, Lane, Clayton-Jones, Thoday and Thomsett reported 5 cases of nasal aspergillosis and compared different diagnostic techniques, with special emphasis on radiology. Their cases were treated by surgery. Following surgery 4 of the cases were infused with nystatin suspension through an in-dwelling tube, one for 3 weeks and the others for 48 hours after surgery. No tube was placed in the 5th case but it was treated with a 2 week course of trimethoprim and sulphadiazine (Tribrissen 80). Thiabendazole was also given orally at a dose rate of 20mg/Kilo per day for 7 days. With the exception of the dog infused with nystatin for 3 weeks, all recovered. In a case described by Chandler (1975) treatment with nystatin was successful without

surgical intervention.

Foster and Stoddart (1976) described a case which they claimed was cured by an autogenous vaccine made from the culture of A. fumigatus isolated from the nasal discharge. Apart from the facts that the vaccine was inactivated at 60°C and suspended in 0.5% Phenol and 0.85% Sodium chloride, details of the technique were not given. These workers did not discuss how the vaccine worked to clear the symptoms or why the dog was cured.

Lane and Warnock (1977) reported the results of examination of a series of 84 dogs with nasal disorders. Of these, 10 were suffering from nasal aspergillosis. The efficiency of various diagnostic methods was compared. The authors found that a combination of the D.D. test for the presence of antibody to A. fumigatus in conjunction with radiology gave a reliable result, but noted that microscopic examination of, or culture from, nasal discharge was not a reliable method.

Barrett, Hoffer and Schultz (1977) investigated the lymphocyte blastogenesis test (L.B.T.) and discovered that their 3 cases showed a very poor response to this test when compared with the controls. Two cases were treated surgically and with 5 fluorocytosine and Sodium iodide and the 3rd with these drugs but without surgery. This dog failed to respond to therapy and was destroyed. The other cases improved clinically but one had a change in the L.B.T. The authors suggested that a possible immunosuppression or an acquired

immunodeficiency could have been present.

Poli, Ponti, Balsari, Addis and Mortellaro (1981) undertook microbiological and serological studies on healthy dogs and on those with nasal aspergillosis. They concluded that the detection of precipitating antibody to A. fumigatus was the most reliable method of confirming a diagnosis of such infection.

However, before one can safely use a serological test in diagnosis of a disease such as nasal aspergillosis it is important to know the proportion of dogs in a normal population which have antibody. A survey was therefore carried out using canine sera referred to the Bacteriology Department. Each serum was tested with 8 antigens prepared from A. fumigatus by D.D. and C.I.E.

To eliminate the possibility that serum antibody found in the first survey had resulted from disease, a second serological survey was undertaken in which examination of nasal passages grossly and by culture was coupled with the serological investigation. The ears of the dogs were also examined and the serum tested for antibody to Pityrosporum pachydermatis, the predominant organism isolated.

In addition, 4 cases of nasal aspergillosis which were treated with ketaconozole were followed clinically and serologically.

ANTIBODY TO A. FUMIGATUS IN A RANDOM SAMPLE OF CANINE SERA.

MATERIALS AND METHODS

The 158 sera investigated for the presence of antibody to A. fumigatus were obtained from the Bacteriology Department of the University of Glasgow Veterinary School, to which they had been sent by veterinary practitioners for screening for bacterial antibodies, or were from "holding" dogs in the Veterinary Hospital.

Brief case histories were available for 130 dogs: 66 were female and 64 male Their ages ranged from 3 months to 13 years. Fifty of the dogs were from urban areas, 73 from suburban areas and 7 were from farms or stables.

Antigens 121, 170, 193 - 197 inclusive and M1 were used to test each serum. Before use in the survey antigens 193 - 197 and M1 were tested using S1, S2 and S3.

All sera were tested by D.D. using pattern C and by C.I.E:

RESULTS

In the preliminary D.D. tests, antigens 193 - 197, as prepared and at a dilution of 1/5, gave positive results with the 3 test sera. The mixed antigen M1 gave a positive result with S1 and S2 but failed to demonstrate antibody in S3.

Precipitating antibody to A. fumigatus was demonstrated in 23 (14.6%) of the 158 canine sera investigated. D.D. tests gave positive results with 9 (5.7%) sera and C.I.E. tests with 23 (14.6%) sera. Of the 23 positive sera, 1 (4.3%) was positive only in the D.D. test, 14 (60.9%) were positive only by C.I.E. and 8 (34.8%) were positive in both tests.

In D.D. tests 8 sera showed a single sharp line and one serum 2 well-defined lines. Five sera gave positive results with 2 antigens and 4 sera with one antigen. Antigen 121 gave positive results with 7 sera, antigen 170 with 3 sera and antigens 193 and M1 with 2 sera each (Table 16). Antigens 194, 195, 196 and 197 failed to demonstrate antibody in any serum.

In C.I.E. tests 2 sera formed 2 lines and the remainder a single line: 6 sera were positive with 4 antigens, 8 sera with 3 antigens, 7 sera with 2 antigens and 2 sera with a single antigen. Antigen M1 gave positive results with 21 sera, antigen 193 with 20 sera, antigen 121 with 16 sera and antigen 170 with 7 sera (Table 17). Antigens 194, 195, 196 and 197 failed to demonstrate antibody in any

serum.

Of the 8 sera positive by D.D. and C.I.E. only 2 gave a positive result with the same antigens, 193 and M1, in each test. None of the 4 sera positive in D.D. with antigen 121 only or the 2 sera positive with antigens 121 or 170 gave a positive result with the same antigen by C.I.E.

Case histories which included ages were available for 126 dogs. The age range was from 3 months to 13 years and details of the number of positive results in the various age groups are given in Table 18. The youngest dog with antibody was 3 months and the oldest was 13. The number of sera in the 5 - 6 year group was, in comparison with the other groups, very low. In the other groups, the proportion of positives increased with age, reaching a peak of 21.4% positive in the 7 - 8 year group and then dropping to 16.7% positive in the oldest group.

The number of male and female animals was almost equal, being respectively 64 and 66; 11 (17.2%) of the males were positive, 5 (7.8%) by D.D. and 11 (17.2%) by C.I.E. Eight (12.1%) of the females were positive, 3 (4.6%) by D.D. and 8 (12.1%) by C.I.E.

In table 19 the results of the serological tests are presented in relation to the environment of the dogs. Of the 7 dogs from farms or stables 4 (57.1%) were positive, 2 by C.I.E. only and 2 by both tests. Of the 8 (11%) positive dogs from country or suburbs 5 were positive only by C.I.E. and 3 by both tests and of the 7

(14%) positive town dogs, 4 were positive by C.I.E. and 3 by both tests.

A brief clinical history was available for 16 of the positive dogs. Only 2 dogs were suffering from nasal disorders, one had a nasal tumour and the other had a persistent nasal discharge. This latter dog was subsequently admitted to the Veterinary School and was found to be free from nasal aspergillosis.

Table 16. Antigens with which canine sera gave a positive reaction in D.D. tests.

Number of sera positive	Antigens			
	121	170	193	M1
2			+	+
3	+	+		
4	+			
9	7	3	2	2

Table 17. Antigens with which canine sera gave a positive reaction in C.I.E. tests.

Number of sera positive	Antigens			
	121	170	193	M1
6	+	+	+	+
8	+		+	+
6			+	+
1	+			+
1	+			
1		+		
23	16	7	20	21

Table 18. The number of dogs examined in the various age groups and the number positive for antibody to A. fumigatus.

Age years	Number of dogs	Number positive		Total positive	
		D.D.	C.I.E.	number	%
<1	24	1	3	3	12.5
1 - 2	16	0	3	3	18.8
3 - 4	15	2	3	3	20.0
5 - 6	21	1	1	1	4.8
7 - 8	14	0	3	3	21.4
9 -10	18	2	3	3	16.7
>10	18	1	3	3	16.7
Total	126	7	19	19	15.0

Table 19. The environments of the dogs and the number of sera from each environment which were positive for antibody to A. fumigatus.

Environment	Number of sera examined	D.D. positive no.	%	C.I.E. positive no.	%	Total positive no.	%
City	50	3	6.0	7	14.0	7	14.0
Suburbs and Country	73	3	4.1	5	6.8	8	11.0
Farms and Stables	7	2	28.6	4	57.1	4	57.1

DISCUSSION

As a result of this survey it has been shown that antibody to A. fumigatus is present in sera from dogs which do not have clinical evidence of nasal aspergillosis. From the number of positive results by D.D. and C.I.E. there is no doubt that the latter is a much more sensitive test for detecting antibody to A. fumigatus in canine sera, a finding which parallels that of MacKenzie and Philpott (1975) for human serum samples. Furthermore, there was also marked similarity in the proportions of sera reacting in each of the tests. Of 106 human sera, 4 (3.7%) were positive only by D.D., 72 (67.9%) only by C.I.E. and 30 (28.3%) by both tests. The corresponding figures in the canine survey were 1 (4.2%), 14 (60.9%) and 8 (34.8%).

No explanation can be given for the rather surprising fact that a few of the canine and human sera were positive by D.D. and negative by C.I.E. MacKenzie and Philpot (1975) suggested that sera stored at -20°C for several months might lose their reactivity and quoted results for a retrospective survey in which 11.26% of sera were positive only by D.D. Unless canine sera differ markedly from human sera when stored at -20°C, this seems unlikely to be the true explanation as many of the canine sera had been held at that temperature for much longer periods of time.

Another fact which cannot be explained in that only 2 of the 8 canine sera positive in both D.D. and C.I.E. gave a positive reaction

with the same antigen in each test.

The use of the D.D. test in the diagnosis of canine nasal aspergillosis was advocated by Lane and Warnock (1977) who found antibody in sera from 10 confirmed cases while sera from 27 control dogs were negative. Poli *et al* (1981), who investigated 15 healthy and 22 diseased dogs, also found antibody to *A. fumigatus* to be present only in sera from diseased dogs and stated that the detection of specific precipitins was the most reliable method of confirming a diagnosis of nasal aspergillosis.

It is true that serological tests play an important part in diagnosis of this disease but, especially now that it has been shown that antibody is present in dogs without clinical signs of the disease, I would stress the importance of interpreting serological results only in conjunction with clinical, radiographical and laboratory evidence.

Antibody to *A. fumigatus* was found in 17.2% of dogs and in 12.1% of bitches. This difference may be a reflection of the habits of the sexes. Dogs usually show a greater tendency to sniff and also tend to roam more than bitches.

The age range of the dogs investigated, from 3 months to 13 years, was sufficiently wide to cover the normal canine life span.

Antibody was found in dogs of all ages; 12.5% in dogs under one year rising to 20% in the 3 - 4 year group. No explanation for the

abnormally low incidence (4.8%) in the 5 - 6 year group can be given, especially as the percentage of positives rose to 21.4% in the next higher age group. From this peak, the percentage of positives then decreased as the age rose.

The most important factor in determining whether an animal will develop antibody is the likelihood of its being exposed to the fungus. As one of the major sources of A. fumigatus is badly made hay, the finding that the group of dogs with the highest proportion of antibody (57.1%) were those associated with farms or stables, was not surprising. The small difference in the proportion of positive animals from town, suburbs and country might be explained by the fact that in the Glasgow district only a very small area in the centre of the city is truly urban. Thus, most city dogs and certainly those from the suburbs would have access to gardens, parks and tracts of wasteland, all sites in which exposure to A. fumigatus would be possible.

One unsatisfactory aspect of this survey was that although brief case histories gave the dog's condition on admission to the hospital or when the serum sample was taken, it was impossible to determine whether any of the positive dogs could have suffered from aspergillosis in the past, were in pre-clinical stages of infection or had merely been exposed to the fungus.

POST MORTEM EXAMINATION OF NASAL PASSAGES AND EARS OF DOGS,

CULTURE OF SAMPLES FROM THESE SITES AND AN INVESTIGATION OF

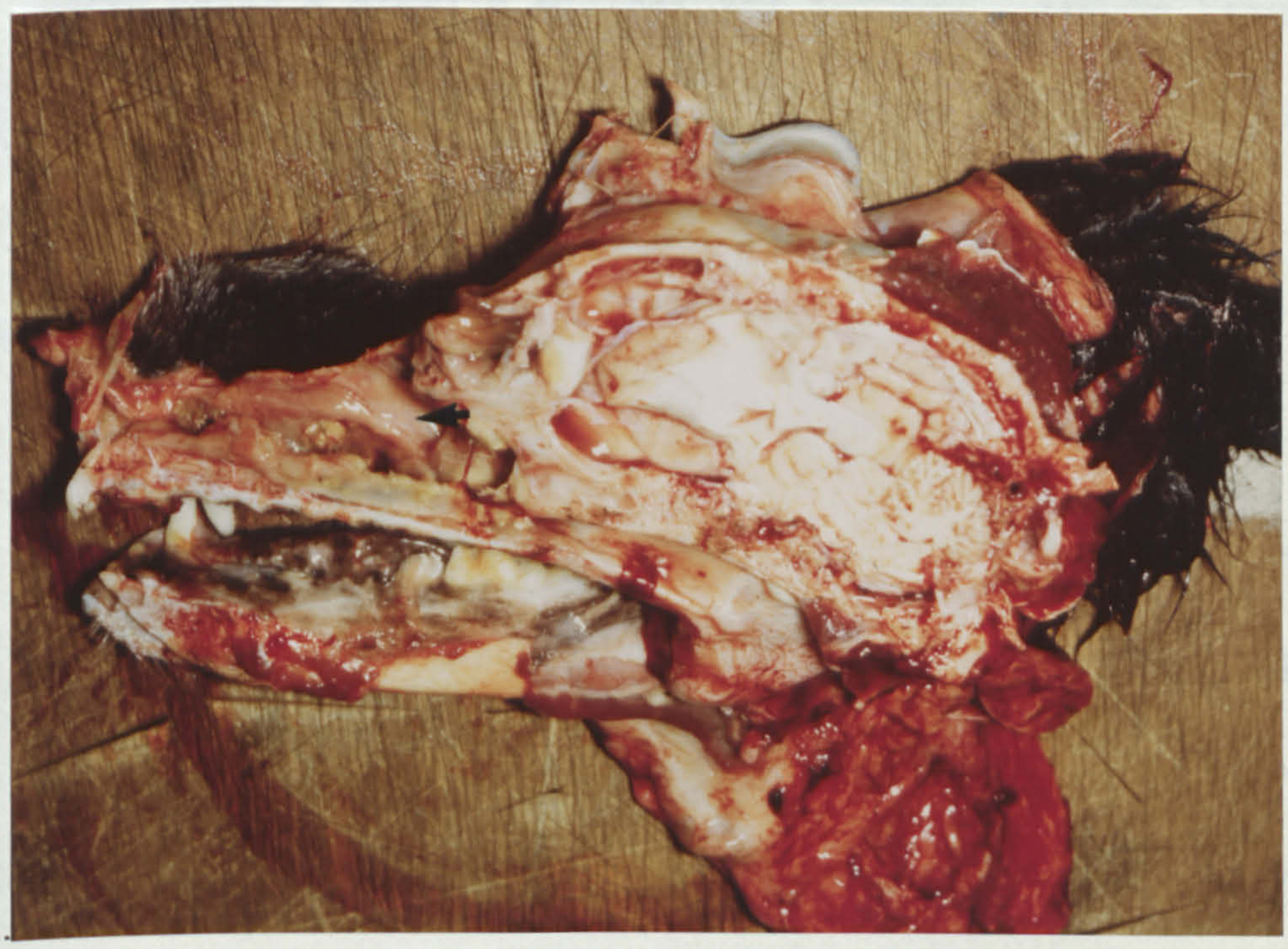
SERUM ANTIBODY TO A. FUMIGATUS AND P. PACHYDERMATIS.

MATERIALS AND METHODS

Of the 102 dogs examined, 86 were obtained from a Dog and Cat home and 16 from the Veterinary School, Glasgow. These dogs had been destroyed for reasons other than disease. The bodies were collected and taken to the post-mortem room as soon as possible after the dogs had been killed, either by an electrical shock or by an overdose of anaesthetic. The time between death and examination was never longer than one hour.

After examination for abnormal conditions, each ear canal was sampled with a sterile cotton swab. The nostrils were examined for evidence of nasal discharge and the antrum of each was sampled by inserting and rotating a swab as far as was possible without using undue force. A blood sample of about 20ml was taken from the jugular vein. The head was than separated from the body and was split with a surgical cleaver along the median plane in such a way that the nasal turbinates were exposed but one set was still covered with its cartilagenous membrane (Fig. 4). This set of turbinates was then opened aseptically, examined for abnormalities and small pieces removed with sterile instruments and placed into sterile Universal bottles.

A smear was prepared from each ear swab and stained by Gram's method. The swab was then used to inoculate a plate of Glucose peptone agar supplemented with chloramphenicol. The plates were



Head of a dog, split open to show the nasal turbinates (arrow).

incubated at 28°C and examined daily for 10 days. Yeasts were identified according to the method of Lodder (1970).

Nasal swabs were inoculated to one plate of Glucose peptone agar and to one of 2% Malt extract agar, each supplemented with chloramphenicol. The plates were incubated at 28°C and examined daily for 15 days.

The samples of nasal turbinates were divided into fragments about 3mm square using a sterile scalpel and forceps. Twelve fragments were inoculated to a plate of each of the 2 media used for isolation from nasal swabs. These plates were also incubated at 28°C and examined daily for 15 days.

Serum was removed from the clotted blood and centrifuged at 3500r.p.m. for 15 minutes. Serum samples were stored at -20°C until used. All sera were tested for the presence of antibody to A. fumigatus using the antigens and techniques for D.D. and C.I.E. described in the previous survey. Sera positive in the D.D. test were titrated against antigen 121 using the method described in the section on antigens.

Each serum was also tested by D.D. for the presence of antibody to Pityrosporum pachydermatis using 9 antigens prepared from 2 strains of this yeast.

Sera from dogs from which Aspergillus sp. other than A. fumigatus were isolated in culture were tested by D.D. with the relevant antigen.

RESULTS

Pathological change was not observed in any turbinate, nor did any dog show signs of nasal discharge.

The results of culture from turbinates and nasal swabs are presented in Table 20. Of 102 dogs examined, 89 (87.3%) yielded one or more fungi on culture; 9 (8.8%) were negative and 4 (3.9%) gave only bacteria. The 89 positive dogs all yielded fungi from the turbinates, 54 (52.9%) from turbinates only and 35 (34.3%) from both turbinates and nasal swab. In no case was the antrum positive and the turbinate negative.

Fungi were isolated from 51 (91.1%) of the 56 adult males and from 28 (87.5%) of the 32 adult females. Of the 35 adult animals in which both turbinate and antrum were positive 23 (41.1%) were male and 12 (37.5%) were female. Of the 54 animals in which only the turbinates were positive, 28 (50.0%) were adult males and 16 (50.0%) were adult females. Ten (71.4%) of the pups gave positive cultures from turbinates, 4 (66.7%) were dogs and 6 (75%) were bitches. In no case was a nasal swab from a pup positive.

Details of the number of animals from which one or more species of fungi were isolated are given in Table 21. From turbinates, 9 (90%) of the 10 positive pups gave one fungus and one (10%) 2; 35 (44.3%) of the 79 adults gave one fungus only, 28 (35.4%) 2, 12 (15.2%) 3 and 4 (5.1%) gave 4. From the antrum 30 (85.7%) of the 35 positive

animals gave one fungus only and 5 (14.3%) 2.

In Table 22 the fungi obtained in culture are listed and the number of animals and the sites from which they were isolated are given. From this table it is apparent that a wider range of fungi was isolated from adult dogs and that the turbinates gave a wider range of fungi than did the nasal antrum. Only 3 genera, Geotrichum, Penicillium and Rhodotorula were recovered with any degree of frequency.

In Table 23 details are given of the number of animals and sites from which these 3 most frequently isolated fungi were recovered in pure culture. From this table it can be seen that isolations of Geotrichum in pure culture far exceeded those of the other 2 fungi.

Species of Aspergillus were isolated only from adult animals and, as will be seen from Table 24, mainly from turbinates. One isolation of A. fumigatus from the nasal antrum of a dog and one of A. niger from the turbinates of a dog were obtained in pure culture. One isolate of A. fumigatus and one of A. flavus were found in conjunction with one other fungus, one A.terreus and 2 isolates of A. niger with 2 other species and one A. terreus and A. niger with 3 other species.

Of 102 sera investigated, 32 (31.4%) were shown to have antibody to A. fumigatus. Details of the sexes and ages of the animals positive by D.D. and/or C.I.E. are given in Table 25 from which it will be seen that 5 (4.9%) of all dogs were positive by D.D. only, 26 (25.5%) by C.I.E. only and one (0.9%) by both tests.

Five (35.7%) pups and 27 (30.7%) adult dogs had antibody. In tests on sera from juveniles and adults, males showed a higher percentage of positives than females. There was a marked difference in the number of positives between the 2 tests when adults and juveniles were compared. By C.I.E. 26.1% and 21.4% of adults and pups respectively were positive whereas by D.D. 3.4% of adults and 14.3% of pups were positive.

The results obtained in D.D. tests with the various antigens are presented in Table 26. Antigen 193 was positive with 4 sera, antigens 194, 196, 197 and M1 with 2 sera each and 195 and 121 with one serum. Antigen 170 was negative with all sera. Sera from 3 adult dogs reacted with one antigen and one serum with 2; one serum from a pup reacted with 3 antigens and the other with 6.

The results obtained in C.I.E. tests with the various antigens are given in Table 27. Antigens 193 - 197 and M1 gave positive reactions with 13 sera each, antigen 170 with 11 sera and 121 with 10. Only antigens 121 and 170 gave a positive reaction with serum from a pup, one serum reacting with both antigens and the other 2 with one only.

Four sera from adult animals reacted with 7 antigens, 9 sera with 6 antigens, 2 sera with 2 antigens and 7 sera with one antigen only.

The one serum positive in D.D. with 121 gave titre 0.

In Table 28 details are given of the fungi isolated from animals positive in the serological tests. Antibody was found in 2 dogs from

which A. fumigatus was isolated and in 2 dogs from which A. niger was recovered. In most of the dogs which had antibody, more than one species was recovered; Geotrichum most frequently, followed by Penicillium and then Rhodotorula.

Of the 102 dogs examined, 22 showed the main clinical signs of otitis externa, exudate and/or excessive wax in the ears. In 8 dogs both ears were involved. Microscopic examination of Gram-stained smears prepared from the ear swabs showed that yeasts were present in the ears of 32 dogs. In 22 dogs, yeasts showing the characteristic morphology of P. pachydermatis were seen; 12 dogs were affected in both ears and 10 in one ear only. Sections of the external auditory canal from a clinically affected dog, stained Gomori-Grocott (Fig.5) and P.A.S. (Fig. 6) revealed typical cells of P. pachydermatis in cellular debris and wax on the surface of the epithelium.

Yeasts other than P. pachydermatis were present in the ears of 10 dogs but only one dog was clinically affected; 2 of these dogs had yeasts in both ears.

P. pachydermatis was isolated from 22 dogs, 12 giving a culture from both ears and 10 from one ear only. Rhodotorula spp. were isolated 8 times, Trichosporon sp. 3 times and Torulopsis once. Species of Candida, other than C. albicans, were recovered from 3 dogs, one of which was clinically positive and yielded the yeast from both ears.

Filamentous fungi were not observed in, or cultured from, any

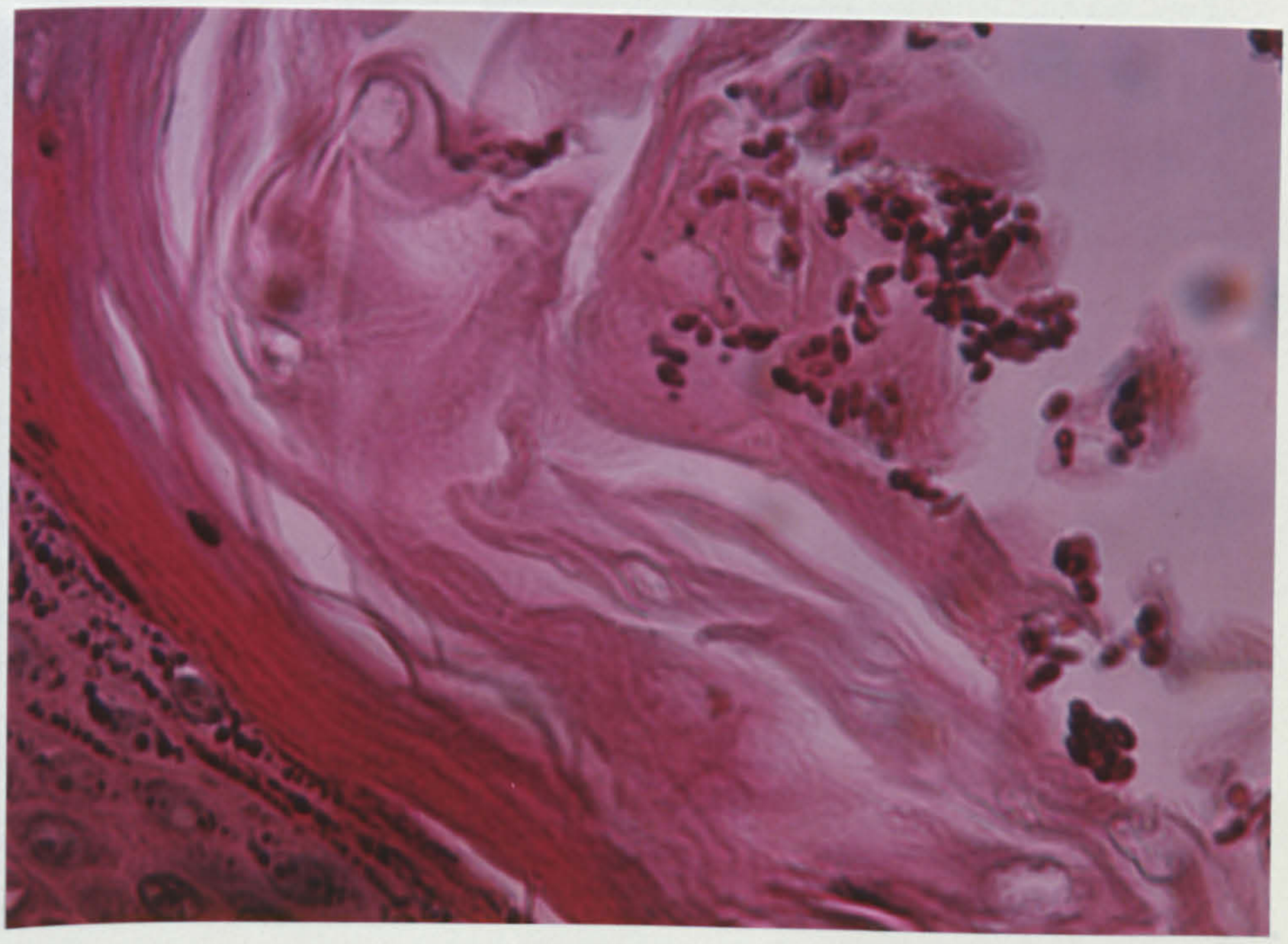


Fig. 5. Section of the external auditory canal of a dog with otitis externa. Cells of P. pachydermatis are present on the surface of the epithelium. Stain, P. A. S. X 250.

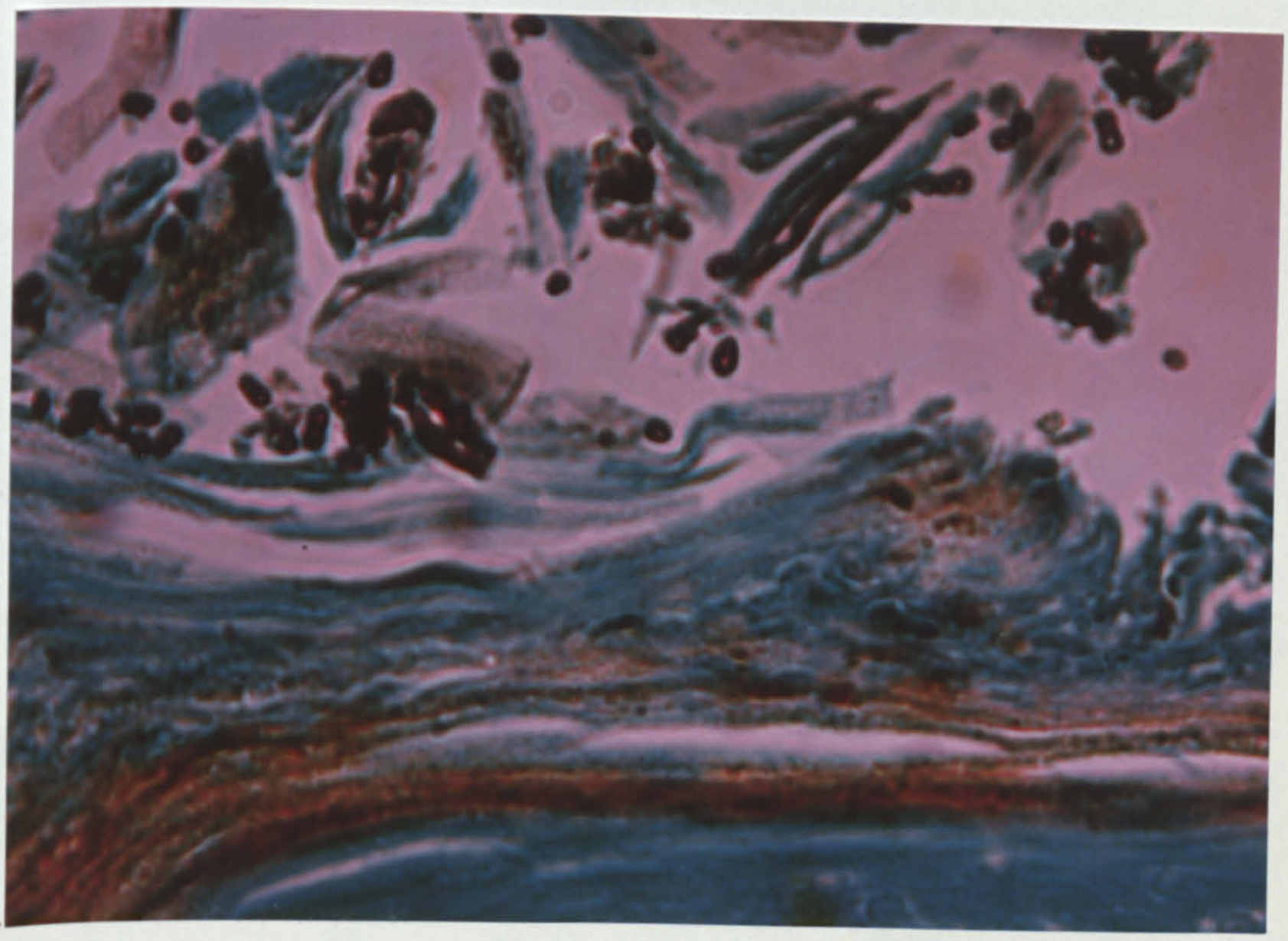


Fig. 6. Section of the external auditory canal of a dog with otitis externa. Cells of P. pachydermatis are present on the surface of the epithelium. Stain, Gomori-Grocott. X 250.

ear swab.

Of the 102 sera examined by D.D. against 9 antigens prepared from P. pachydermatis, 2 gave a positive reaction of a single sharp line. One positive serum was from a dog with otitis due to P. pachydermatis and the other was from a dog with apparently normal ears. Neither of these serum samples was positive for antibody to A. fumigatus.

Table 20. Details of the number of dogs from which turbinates and nasal swabs were examined by culture and the numbers from which fungi were isolated.

Dog. Age and sex	Number investigated	Positive					
		turbinates only		turbinates and nasal swabs		total	
		no.	%	no.	%	no.	%
Puppy dog	6	4	66.7	0	0	4	66.7
Puppy bitch	8	6	75.0	0	0	6	75.0
Total puppies	14	10	71.4	0	0	10	71.4
Dog adult	56	28	50.0	23	41.1	51	91.1
Bitch adult	32	16	50.0	12	37.5	28	87.5
Total adults	88	44	50.0	35	39.8	79	89.8
Total all dogs	102	54	52.9	35	34.3	89	87.3

Table 21. Details of the number of dogs and the sites from which one or more fungal genera were isolated.

Dog age & sex	Turbinates					Nasal antrum				
	total	number of genera				total	number of genera			
	+	1	2	3	4	+	1	2	3	4
Puppy dog	4	4 100%	0	0	0	0	0	0	0	0
Puppy bitch	6	5 83.3%	1 16.7%	0	0	0	0	0	0	0
Puppy total	10	9	1	0	0	0	0	0	0	0
Dog adult	51	26 51.0%	14 27.5%	8 15.7%	3 5.9%	23	20 87.0%	3 13.0%	0	0
Bitch adult	28	9 32.1%	14 50.0%	4 14.3%	1 3.6%	12	10 83.3%	2 16.7%	0	0
Adult total	79	35 44.3%	28 35.4%	12 15.2%	4 5.1%	35	30 85.7%	5 14.3%	0	0

Table 22. Details of the dogs and sites from which the various fungi were isolated.

Fungus	Turbinates					Nasal antrum *		
	Puppy		Adult		Total	Adult		Total
	dog	bitch	dog	bitch		dog	bitch	
Aspergillus	0	0	5	2	7	1	1	2
Geotrichum	0	1	28	14	43	8	7	15
Penicillium	0	1	23	12	36	8	1	9
Rhodotorula	1	2	15	16	34	2	1	3
Mucor	1	2	2	1	6	0	1	1
Aleurisma	1	0	3	0	4	2	1	3
Black yeasts	0	0	3	1	4	0	0	0
Other yeasts	2	0	2	3	7	3	1	4
Fusarium	0	0	2	0	2	1	0	1
Tubercularia	0	0	1	1	2	0	0	0
Mortierella	0	0	1	1	2	0	0	0
Cladosporium	0	0	0	0	0	1	0	1
Isaria	0	0	0	1	1	0	0	0
Hyphomyces	0	0	1	0	1	0	0	0
Paecilomyces	0	0	1	0	1	0	0	0
Scopulariopsis	0	0	1	0	1	0	0	0
Trichosporon	0	0	1	0	1	0	0	0
Non-sporing dematiaceous	0	0	0	2	2	0	0	0

* No pup yielded fungus from nasal antrum

Table 23. Details of the animals and the sites from which Geotrichum, Rhodotorula and Penicillium were isolated in pure culture.

Site	Dog	Geotrichum	Rhodotorula	Penicillium
Turbinates	Pup dog	1	1	0
	Pup bitch	0	1	1
	Adult dog	13	3	7
	Adult bitch	4	2	0
Nasal antrum	Pup dog	0	0	0
	Pup bitch	0	0	0
	Adult dog	7	0	3
	Adult bitch	6	1	0

Table 24. The species of Aspergillus isolated and the sites from which they were recovered.

Species	Turbinates		Nasal antrum		Total
	Dog	Bitch	Dog	Bitch	
A. fumigatus	1		1		2
A. terreus	1	1			2
A. niger	3	1			4
A. flavus				1	1
Total	5	2	1	1	9

Table 25. Details of the number of sera investigated by D.D. and C.I.E. from adult and juvenile dogs and the result of the tests.

Age and sex	Number of sera tested	Positive							
		D.D. only		C.I.E. only		Both tests		Total	
		no.	%	no.	%	no.	%	no.	%
Pup dog	6	1	16.7	2	33.3	0	0	3	50
Pup bitch	8	1	12.5	1	12.5	0	0	2	25
Pup total	14	2	14.3	3	21.4	0	0	5	35.7
Adult dog	56	2	3.6	17	30.4	1	1.8	20	35.7
Adult bitch	32	1	3.1	6	18.8	0	0	7	21.9
Adult total	88	3	3.4	23	26.1	1	1.1	27	30.7
All dogs total	102	5	4.9	26	25.5	1	0.9	32	31.4

Table 26. Details of numbers of sera from adult and juvenile animals which reacted with the antigens in the D.D. tests.

Dog age	Number of sera +ve	Antigens							
		121	170	193	194	195	196	197	M1
Pup	1				+	+			+
	1				+	+	+	+	+
Adult	2				+				
	1	+							
	1						+	+	
Total	6	1	0	4	2	1	2	2	2

Table 27. Details of numbers of sera from adult and juvenile animals which reacted with the antigens in C.I.E. tests.

Dog age & sex	Number of sera +ve	Antigens							
		121	170	193	194	195	196	197	M1
Pup	1	+							
	1	+	+						
	1		+						
Adult bitch	1	+		+	+	+	+	+	+
	1		+	+	+	+	+	+	+
	2	+							
	2			+	+	+	+	+	+
Adult dog	2	+							
	5		+						
	2	+	+						
	1	+		+	+	+	+	+	+
	1		+	+	+	+	+	+	+
	7			+	+	+	+	+	+
Total	27	10	11	13	13	13	13	13	13

Table 28. Details of the animals positive in serological tests and the fungi which were isolated from them.

Test	Dog	No. + serol.	No. + culture	No. -	Aspergillus	Pen.	Geo.	Kho.	Other fungi
D.D.	Pup	2	2	1				+	
				1					+
	Adult	4	4	1	fumigatus	+			+
				1	niger				
				1		+	+		+
				1			+		
Total		6	6	6	2	2	2	1	3
C.I.E.	Pup	3	2	2			+		
	Adult	24	20	1	fumigatus		+		
				1	niger	+		+	
				3		+		+	
				2			+	+	+
				5			+		
				2		+	+	+	
				2		+	+		
				1		+			
				1		+			+
				1		+	+		+
				1		+			+
Total		27	22	22	2	12	15	8	5

DISCUSSION

Turbinates proved to be a more fruitful source of fungi than the nasal antrum. This may have been due to the sampling techniques used since swabs were taken from the antrum and fragments of tissue from the turbinates. It could be, however, that clearance of fungi from the antrum is rapid and that those present were ones to which the dog had been recently exposed. Alternatively, it might be that as the dog lacks nasal hairs, inhaled fungal elements might pass through the antrum without being trapped and be deposited on the mucous membranes of the turbinates.

The range of fungal genera isolated from the 2 sites was limited and only Geotrichum, Rhodotorula and Penicillium were recovered with any frequency. Species of Aspergillus were not common and A. fumigatus was recovered only from 2 samples. There was a difference in the prevalence of fungi in adult and juvenile animals and in dogs and bitches, for which no satisfactory explanation can be given.

Filamentous fungi were not observed on microscopy and were not obtained in culture from ear samples. It would appear therefore that, unlike man in whom Aspergillus spp. are a major cause of otitis externa, this genus is of no importance in the dog. Of the yeasts isolated from ear swabs, P. pachydermatis was the dominant species.

In this survey, 31.4% of the dogs investigated were shown to have antibody to A. fumigatus, 5.9% by D.D. and 26.5% by C.I.E. Four of the 9 dogs from which Aspergillus sp. were isolated gave a positive

reaction. Two of these positive animals were those from which the 2 isolations of A. fumigatus were made. None of the serum samples from dogs from which other species of Aspergillus were recovered were positive in D.D. tests with the antigen prepared from the relevant species.

Cross reactions between A. fumigatus and other species in the genus have been demonstrated by a number of workers using hyperimmune serum raised in rabbits. With the antigens used routinely in the laboratory (121, 170) and the others prepared for the serological surveys, no cross reactions to other species of Aspergillus, namely nidulans, terreus and flavus raised in rabbits. Biguet, Tran van Ky, Andrieu and Fruit (1964) found no evidence of cross reaction with Geotrichum or with Candida, slight reaction with Mucor mucedo and with Trichophyton soudanense, a species which does not occur in dogs in this country. Three pups in this series gave Mucor sp. from the turbinates and were also positive for antibody to A. fumigatus. It is likely that they were truly positive to A. fumigatus as they had come from a farm and also, had been held in the animal house at the Veterinary School for several weeks before being destroyed.

As Penicillium spp. were among the commonest of the isolates, the possibility of cross reaction must be considered. However, Harvey, O'Brien, Felsburg, Izenberg and Goldschmidt (1981) investigated sera from 2 dogs with nasal penicillosis, using Penicillium-mixed allergenic extracts as antigen and also tested the sera with A. fumigatus antigen.

They found that both sera reacted only with the Penicillium antigen. It therefore seems unlikely that cross reactions with Penicillium would be picked up in D.D. tests in the survey.

Some differences in the results obtained from this and the previous survey were apparent. In the first survey a total of 14.6% of the dogs were positive for antibody to A. fumigatus compared with 31.4% in the second survey. There was a marked difference in the number of positives obtained in C.I.E. tests in the 2 surveys, 14.6% and 26.5% respectively, but there was good correlation between the results from the D.D. tests (5.7% and 5.9%).

Perhaps exposure of the dogs to A. fumigatus in the 2 surveys was different. The sera in the first survey were taken in a different year and throughout the year, while those in the second survey were collected between July and November. According to Hudson (1969) who studied Aspergillus spp. in the air spora at Cambridge, the months in which Aspergillus spp. reach a peak are November and December and, in a second paper (1973) on thermophilous and thermotolerant fungi in the atmosphere, he found A. fumigatus to be the most frequent species, with the highest numbers occurring from November to January with another peak in June. However, if increased exposure were the explanation for the higher number of positives in the second survey, one would have expected variation in the D.D. tests as well as those from C.I.E.

It could be possible that because most of the dogs in the first survey were suffering from some disease condition, some of them might

have a reduced immune response. Also, as they were unwell they perhaps went out less and so were less exposed to the fungus. As many of the dogs in the second survey came from a Dog and Cat Home, some of them would have been strays and could have been exposed to A. fumigatus while foraging for food. Again, however, these explanations seem unlikely because of the results of the D.D. tests.

A more likely explanation for the discrepancy in the results is that the first set of sera had been stored at -20°C for at least 6 months and, during that time had probably been thawed several times before being examined for antibody to A. fumigatus. MacKenzie and Philpot (1975) suggested that human sera stored at this temperature for several months would lose reactivity in C.I.E. tests. C.I.E. is generally considered to be a very sensitive test and by using it one can detect much smaller amounts of antibody than can be detected by D.D. Thus, sera positive in C.I.E. and negative in D.D. might be assumed to have less antibody than sera positive by D.D. and therefore might well be those which would lose their reactivity more readily. On re-testing old sera therefore one might expect that sera originally positive in D.D. tests would be more liable to give a positive in C.I.E. than sera originally positive only by C.I.E.

If antibody to A. fumigatus in a canine population is relatively constant, as would appear to be the case from the consistent results in D.D. obtained from the 2 surveys, and if the difference in the C.I.E. results is due to the effects of storage, on re-testing old sera one

would expect no alteration in the D.D. test results and a drop in the number of sera positive by C.I.E.

The old and new canine sera reacted differently with the various antigens, the old sera giving positive results with fewer antigens than did the new sera. This suggests that the ability to react with certain antigens may vary with the storage life of a serum. Of the antigens which reacted with old sera, 121 and 170 were culture filtrate antigens prepared from multiple strains of A. fumigatus; 193 was a culture filtrate antigen prepared from a single strain on Y.M. medium which, in the first part of this thesis, was shown to be an excellent medium for the production of antigens. M1 was a mixture of antigens 1 193 - 197. It therefore looks as if multiple antigens, or very good single antigens are best for use with old sera.

Biguet, Tran van Ky, Capron and Fruit (1962) have shown that A. fumigatus can produce up to 25 antigenic fractions capable of causing the production of antibody. It might be assumed that these serum antibodies would not all have equal life spans on storage, especially if sera were subjected to freezing and thawing. It may be therefore that the multiple antigens, which might be expected to react with a wider range of antibodies would be more likely to be effective with old sera than single strain antigens. However, to prove this very tentative hypothesis, it would be necessary to carry out controlled experiments.

As a result of this survey it has been proved that antibody to

A. fumigatus is present in a proportion of sera from dogs with no evidence of present or past nasal aspergillosis.

CANINE NASAL ASPERGILLOSIS

CASE STUDIES

INTRODUCTION

Dawson et al (1973) reported the first case of nasal aspergillosis in dogs admitted to the Veterinary School of the University of Glasgow; since then, 21 further cases have been admitted. Only in the last year has systematic follow-up of the serum samples from cases been possible. Veterinarians routinely submit cases for diagnosis of nasal aspergillosis. The dogs are seen by the clinicians who send samples to the mycology laboratory. A D.D. test for A. fumigatus antibody is performed on the serum of the patient. Culture of nasal washings and/or swabs is carried out and this material is also examined by microscopy. The culture results from swabs depends mainly on the ability of the clinician to observe the plaque-like lesion with an endoscope and take the sample directly from this area. This is sometimes impossible to achieve because of the site of the lesion and in these cases culture results from swabs are poor.

The results are sent back to the clinician in charge of the case but in only a proportion of the cases is the mycology laboratory informed of the progress of the dog. This may be because the dog is destroyed or is sent home to be treated by the local veterinarian. When the clinician decides to attempt treatment this is probably surgical. The mycology laboratory will then be involved in the case again when material removed from the nasal turbinates or sinuses is sent for examination.

I was involved in following 4 cases of canine nasal aspergillosis admitted to the Veterinary Hospital during a period of 7 months. These cases were diagnosed clinically by the Surgery Department and were then used in a drug trial with Ketoconazole (Janssen Pharmaceutical Research Laboratories).

MATERIALS AND METHODS

Antigens. A. fumigatus 121, 170, M1

Serological tests. D.D. was performed using pattern B. C.I.E. was performed using the standard method. Sera were titrated with antigen 121 in both tests.

Mycological investigations. Samples from surgery were mounted in 20% KOH and examined microscopically and about 12 small fragments were inoculated to 2% Malt extract agar supplemented with Chloramphenicol and incubated at 37°C.

The dogs were followed clinically and where post mortem examination was performed, the pathologist in charge was asked to look specifically for signs of spread of the infection. Samples were taken for sectioning and the sections were stained with H and E, P. A. S., and Gomori-Grocott.

To obtain more information on serology of the disease and the use of D.D. and C.I.E. in diagnosis, further studies were carried out on each case. Six samples were also investigated by I. M. E.

Case 1

A male 2½ year Alsatian was first seen by the clinician in September, 1981. It presented with left-sided mucohaemorrhagic nasal discharge and discomfort and pain in the nose. In December 1981, the dog was admitted to the Veterinary Hospital; the main clinical sign was the left-sided haemorrhagic nasal discharge. X-ray examination revealed opacities in the left frontal sinus and a serum sample showed antibody to A. fumigatus at a titre of 1/8 by D.D. The dog suffered a severe bout of epistaxis over Christmas and in January a left-sided rhinotomy to remove the infected turbinates was performed. A. fumigatus was cultured from the material removed and the titre of a serum sample was 1/2. Treatment with Thiabendazole at 20mg/Kg/day was started. After one month the left-sided nasal discharge was still present, the dog was losing weight and the titre had risen to 1/8. In March, the treatment with Thiabendazole was stopped and Ketoconazole was started on the 2nd at a dose of 40mg/Kg/day. From that time blood samples were examined weekly (Table 29). At the start of treatment the titre was 1/4, fell to 0 by 10th March, rose to 1/16 by the 17th and fell to 1/8 by the 24th. On the 10th March X-rays still revealed opacities on the left frontal sinus. Exploratory surgery was performed and the material from this was positive on microscopic examination (Fig. 7) and A. fumigatus was obtained in culture. By April 1982, X-ray plates and examination by endoscopy failed to reveal any sign of aspergillosis and culture of a biopsy sample was negative. The dog was sent home

Fig. 7. Immature and mature conidial heads of A. fumigatus in a KOH preparation of material removed from case 1 at operation.

on the 10th April still on treatment with Ketoconazole at half dose for a further week. The titre had fallen to 1/2 by the 26th. April. By May, the dog was eating well, there was no nasal discharge and X-ray plates were clear. The serum titre had remained at 1/2 but by June it was down to 0 and the dog remained without any signs of illness. This dog is still alive and well.

Case 2

A female 21 month old Golden Retriever, presented with unilateral left-sided, blood-stained nasal discharge of 3 weeks' duration. Soft tissue swelling was observed over the left frontal sinus area and nasal aspergillosis was diagnosed in the left nasal cavity and left frontal sinus by radiography, endoscopy and serology (D.D. titre 1/2). On the 5th of May treatment with Ketaconazole was started at a rate of 40mg/Kg/day. Weekly blood samples were obtained (Table 29). The titre at the start of treatment was 1/2, rose to 1/4 on the 12th and to 1/32 on the 19th. Previously, on the 11th exploratory surgery had been performed on the left frontal sinus, revealing numerous plaque-like colonies. The material removed showed characteristic conidial heads of A. fumigatus in KOH preparations (Fig. 8) and branching septate hyphae (Fig. 9).

By June the condition of the dog had deteriorated and the nasal discharge was more pronounced. The left sinus was re-opened and an extensive plaque of fungal growth was observed. The frontal and orbital bones were extensively involved with much destruction. The

Fig. 8. Conidial head of A. fumigatus in a KOH preparation of material removed from the nasal turbinates of case 2 by surgery.

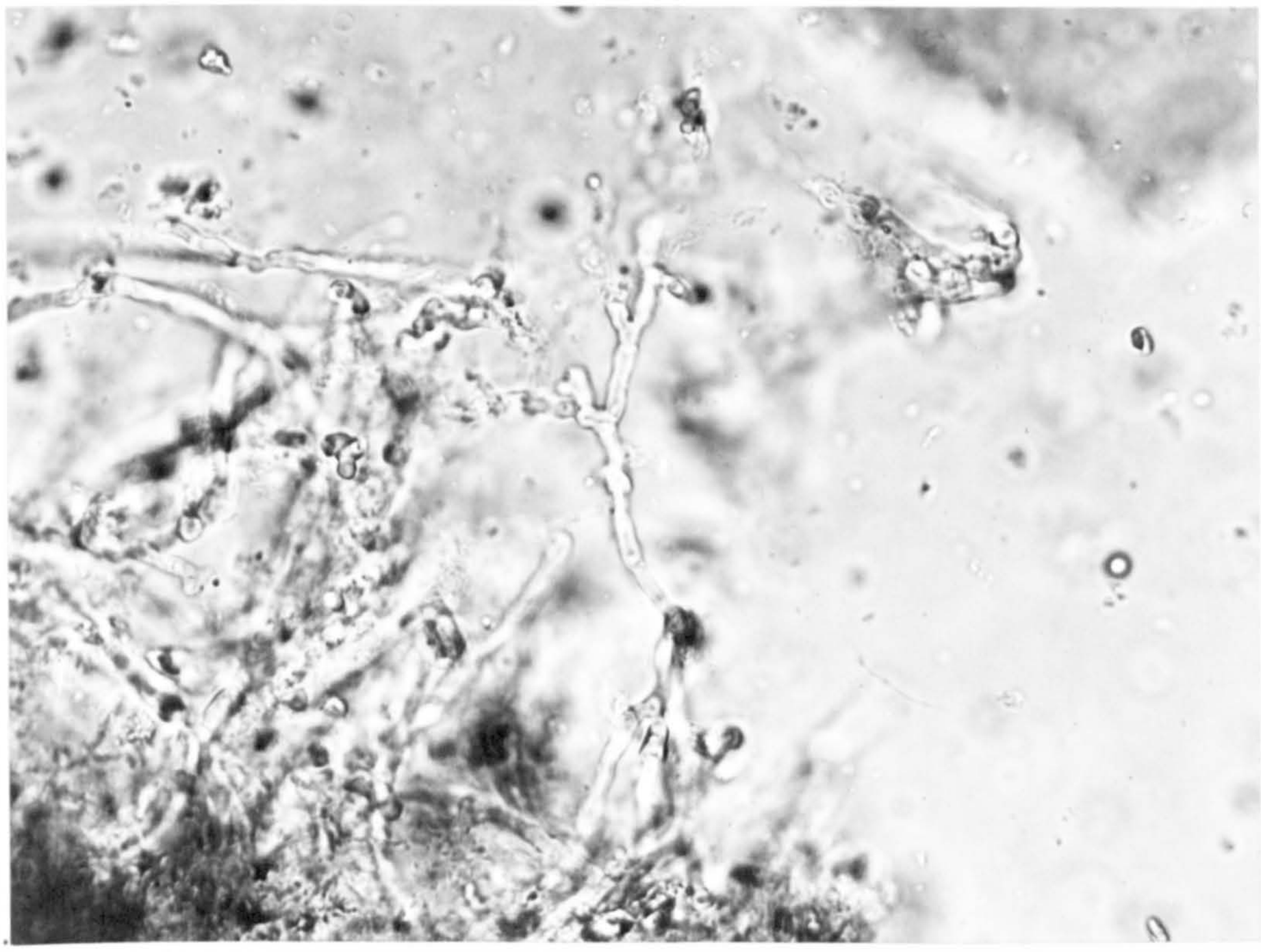


Fig. 9. Branching septate hyphae in a KOH preparation of material removed from the nasal turbinates of case 2 by surgery. X 250.

serum titre was 1/16. Radiography, including venogram, confirmed bone destruction and suggested that the tempero-mandibular joint was also involved. The dog, at this time was dull and in some discomfort. On the 4th June it was destroyed and examined post mortem. The titre then was 1/32.

The results of the post mortem examination confirmed necrotising rhinitis involving the left nasal cavity and adjacent structures. The regional lymph nodes were enlarged. There was no evidence of spread to the cranial cavity. Microscopical examination of sections confirmed the presence of necrotising rhinitis characterised by proteinaceous debris with an increase in polymorphs. Fungal elements were seen in the debris (Figs 10, 11). Sections of the zygomatic arch revealed a chronic inflammatory reaction in adjacent tissues and some evidence of bone necrosis with periostial proliferation. Fungal elements were not observed in these sections. Sections from the left eye and orbital tissues revealed a chronic inflammatory reaction extending to the sclera and conjunctiva but the substance of the eye appeared unaffected. Local lymph nodes showed mild reactive hyperplasia with active germinal centres. There were no other significant findings in this case.

Case 3

A female 2 year, Old English Sheepdog was referred with loss of appetite and a left-sided purulent, blood-stained nasal discharge of 7 weeks' duration. The condition had shown no response to antibacterial

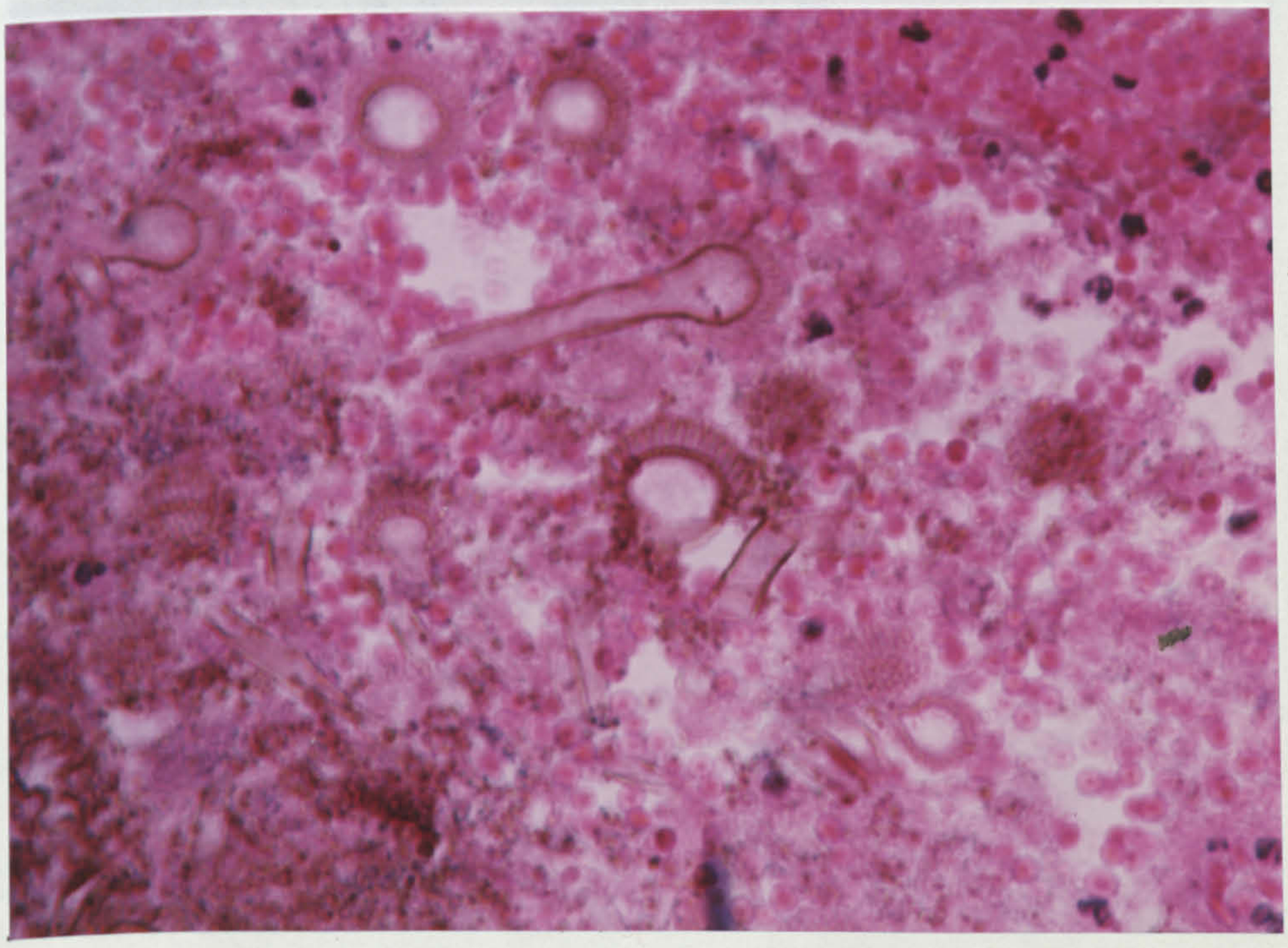


Fig. 10. Section through a lesion from a case of necrotising rhinitis showing conidial heads of A. fumigatus within cellular debris. Stain, P. A. S., X 250.

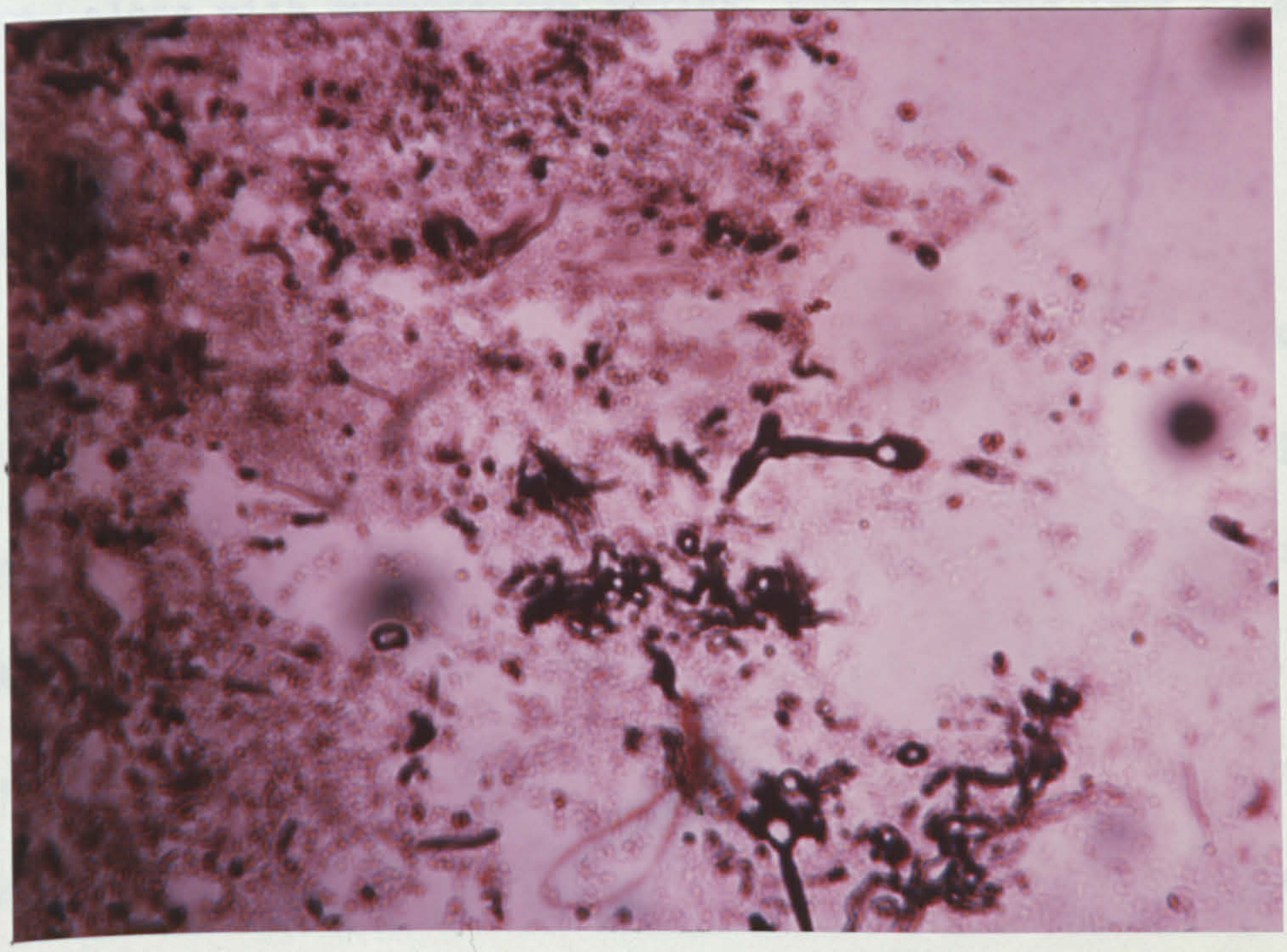


Fig. 11. Section through a lesion from case 2 showing hyphae and conidial heads of A. fumigatus within necrotic debris. Stain, Gomori-Grocott, X 100.

antibiotics. Diagnosis of nasal aspergillosis was made on the basis of radiography, endoscopy and a positive D.D. test with a titre of 1/32. The dog was bright, alert and non-pyretic. Treatment with Ketoconazole at 40ml/Kg/day was commenced on the 12th April immediately following exploratory surgery to establish the presence of Aspergillus sp. The radiographic and endoscopic picture remained unaltered. A. fumigatus, along with mucoraceous moulds, was cultured from nasal washings and, at this time the titre was 1/8. The dog had gained weight and was improving in condition. On the 16th May it died suddenly overnight, having shown no abnormal clinical signs the previous evening. Unfortunately it was impossible to carry out a satisfactory post mortem examination due to advanced post mortem autolysis. Significant findings were a haemorrhagic nasal discharge and congestion and haemorrhage in the nasal and ethmoidal turbinates. An interesting finding in this case was that septate hyphae were seen in mounts prepared from the spinal cord and A. fumigatus was cultured from this material.

Case 4

A male 12 year old mongrel was admitted in May 1982 showing a bilateral haemorrhagic and mucopurulent nasal discharge. The dog was very dull and inappetent. Radiography showed loss of maxilloturbinate patterns in both nasal passages and increased soft tissue density in the posterior nasal chambers, suggestive of aspergillosis. The diagnosis

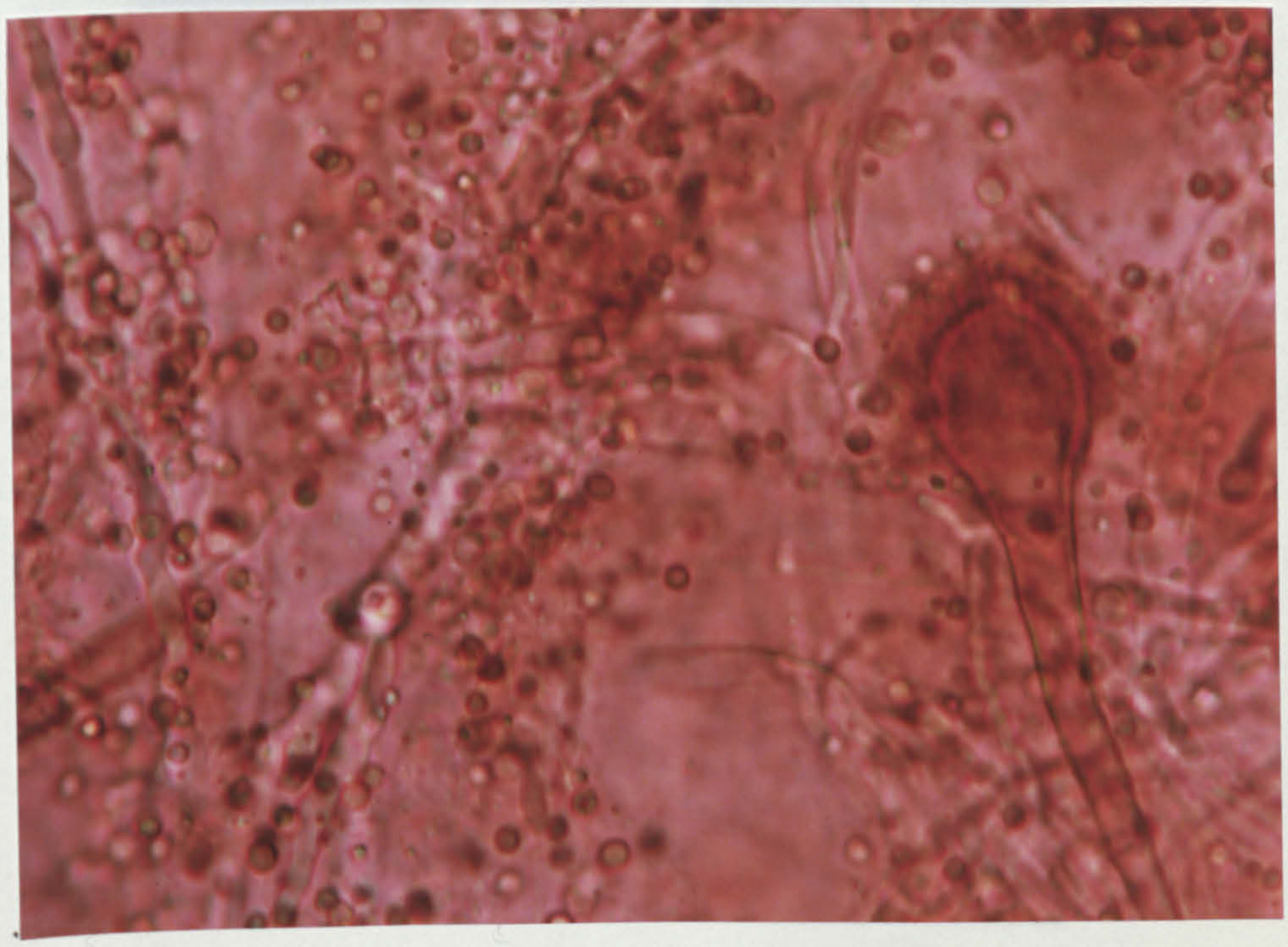


Fig. 12. Hyphae, spores and a conidial head of A. fumigatus in a KOH preparation of material removed from Case 4 by surgery. X 500.

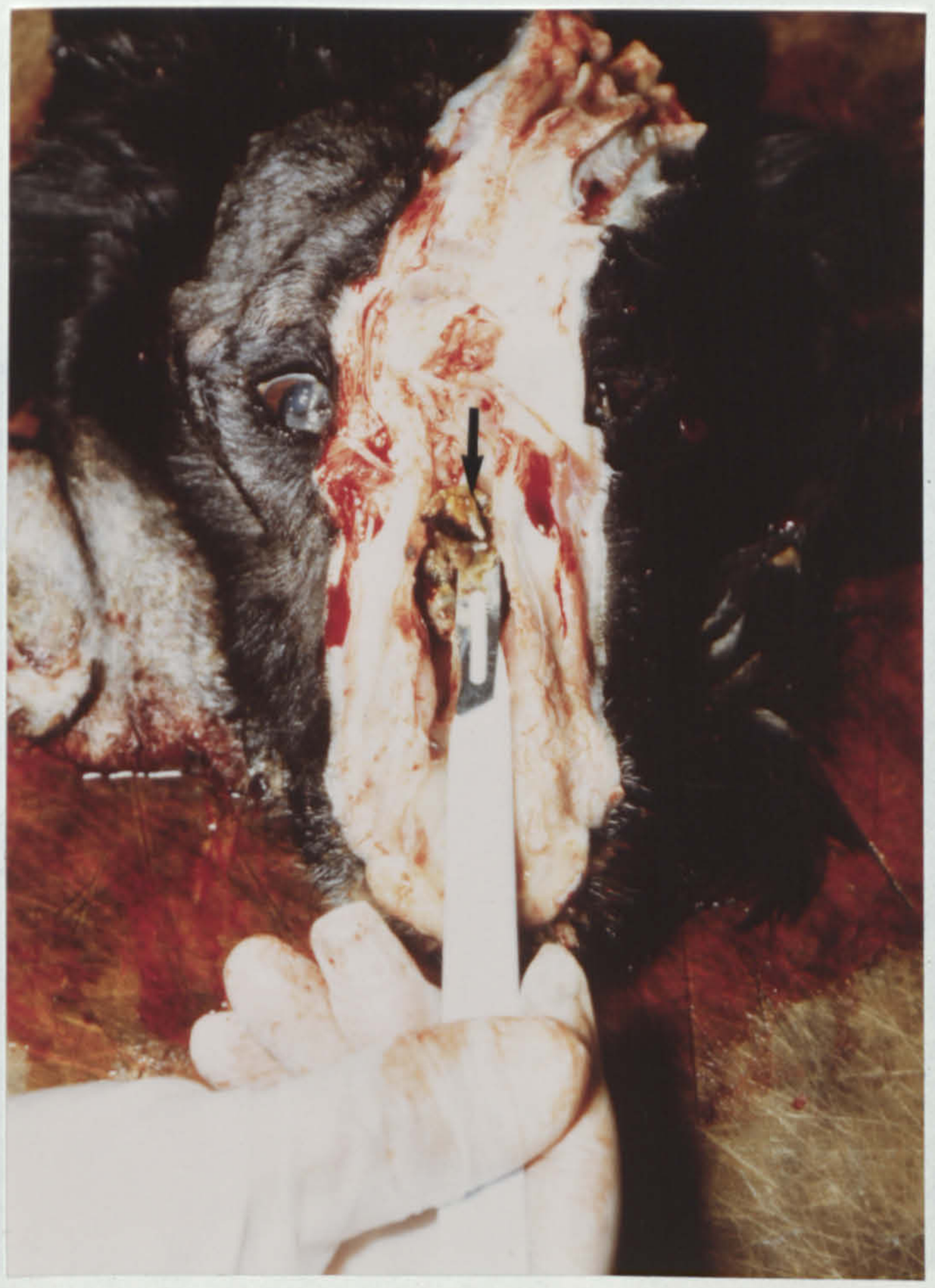


Fig. 13. Head of a dog opened to show the lesions (arrowed) of nasal aspergillosis caused by A. fumigatus.

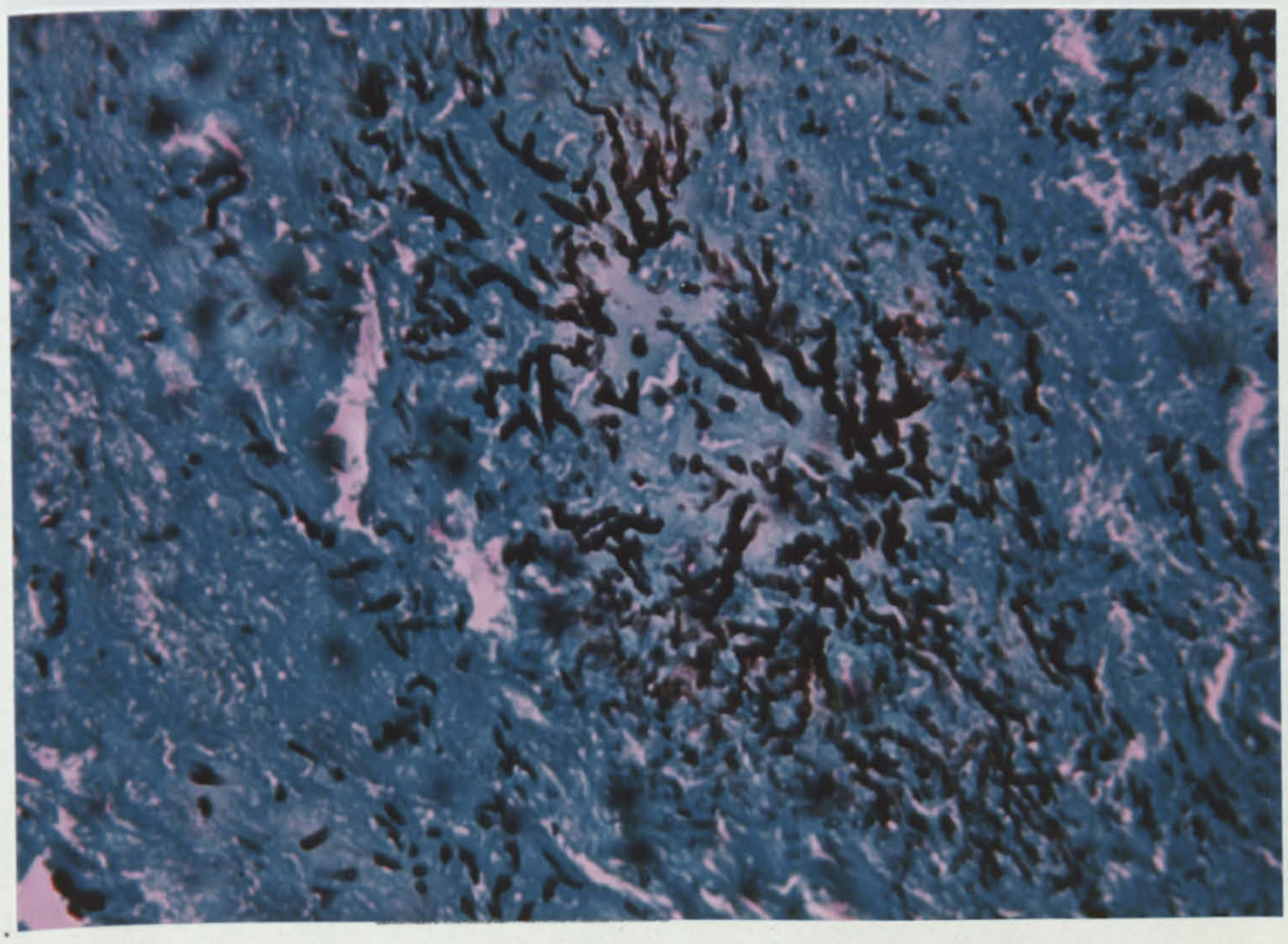


Fig. 14. Section through a small fungal colony within necrotic debris.

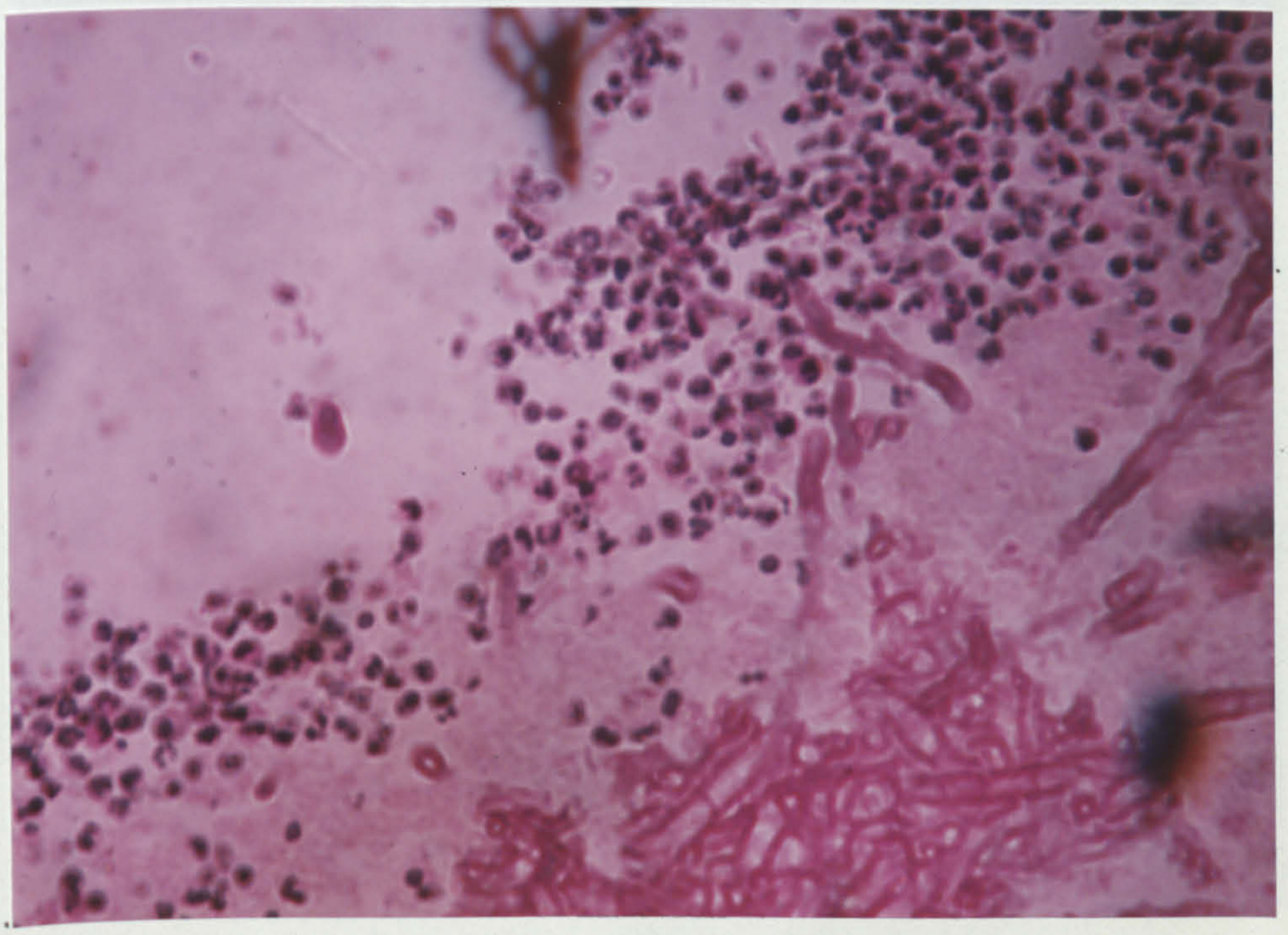


Fig. 15. Section of lesion from nasal turbinates showing a densely interwoven mass of septate hyphae surrounded by an intense cellular reaction. Stain, P.A.S. X 250

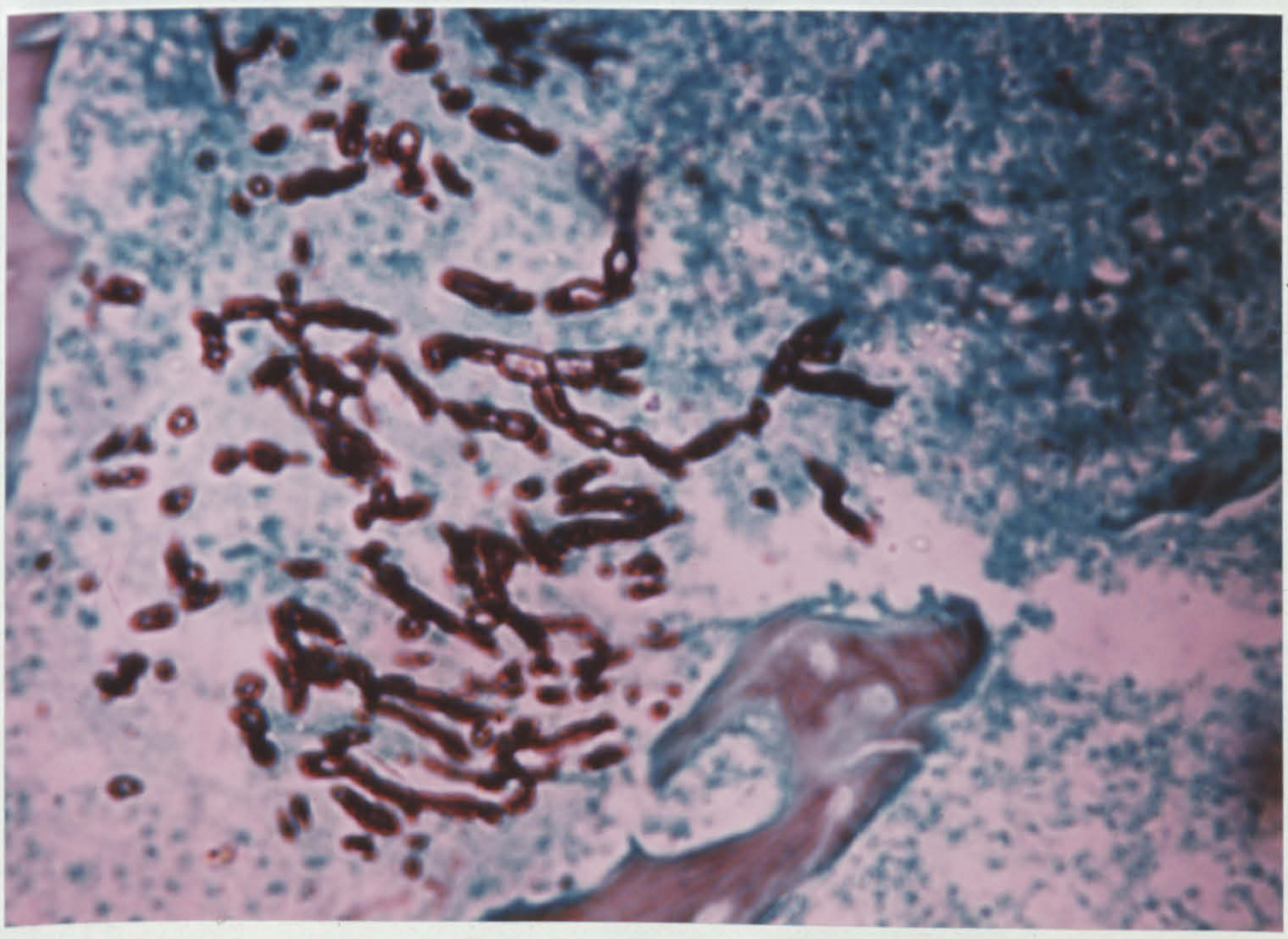


Fig. 16. Section of lesion from nasal turbinates showing branching septate hyphae within necrotic debris. Stain, Gomori-Grocott, X 250.

was confirmed by a serum titre of 1/16, on the 11th May. Treatment with Ketoconazole at 40mg/Kg/day was started on this date. Exploratory surgery was carried out on the 17th and a walnut-sized lesion was removed from the right nasal cavity. Septate hyphae and conidial heads were observed in KOH preparations from this material (Fig. 12) and A. fumigatus was obtained in culture. Unfortunately, the dog could not tolerate the drug. Its condition deteriorated rapidly and it was destroyed on the 2nd June. On this date the serum titre was 1/8.

At autopsy, a severe necrotising rhinitis with destruction of the nasal turbinates was evident (Fig. 13). The necrotic debris contained small yellow foci which, in section, proved to be small fungal colonies (Fig. 14). The submandibular and retropharyngeal lymph nodes appeared enlarged and reactive. The liver was rather small and appeared firm and granular; a discrete tumour of approximately 3cm in diameter protruded from the right lobe. Additional findings were mild endocarditis of the left atrioventricular valve and a small, firm and mottled prostate gland.

Microscopy of sections confirmed the presence of necrotising rhinitis with branching septate hyphae in the necrotic debris (Figs. 15, 16). Sections of the liver revealed diffuse hepatocellular degeneration superimposed on the typical hyperplastic nodular structure of an old dog's liver.

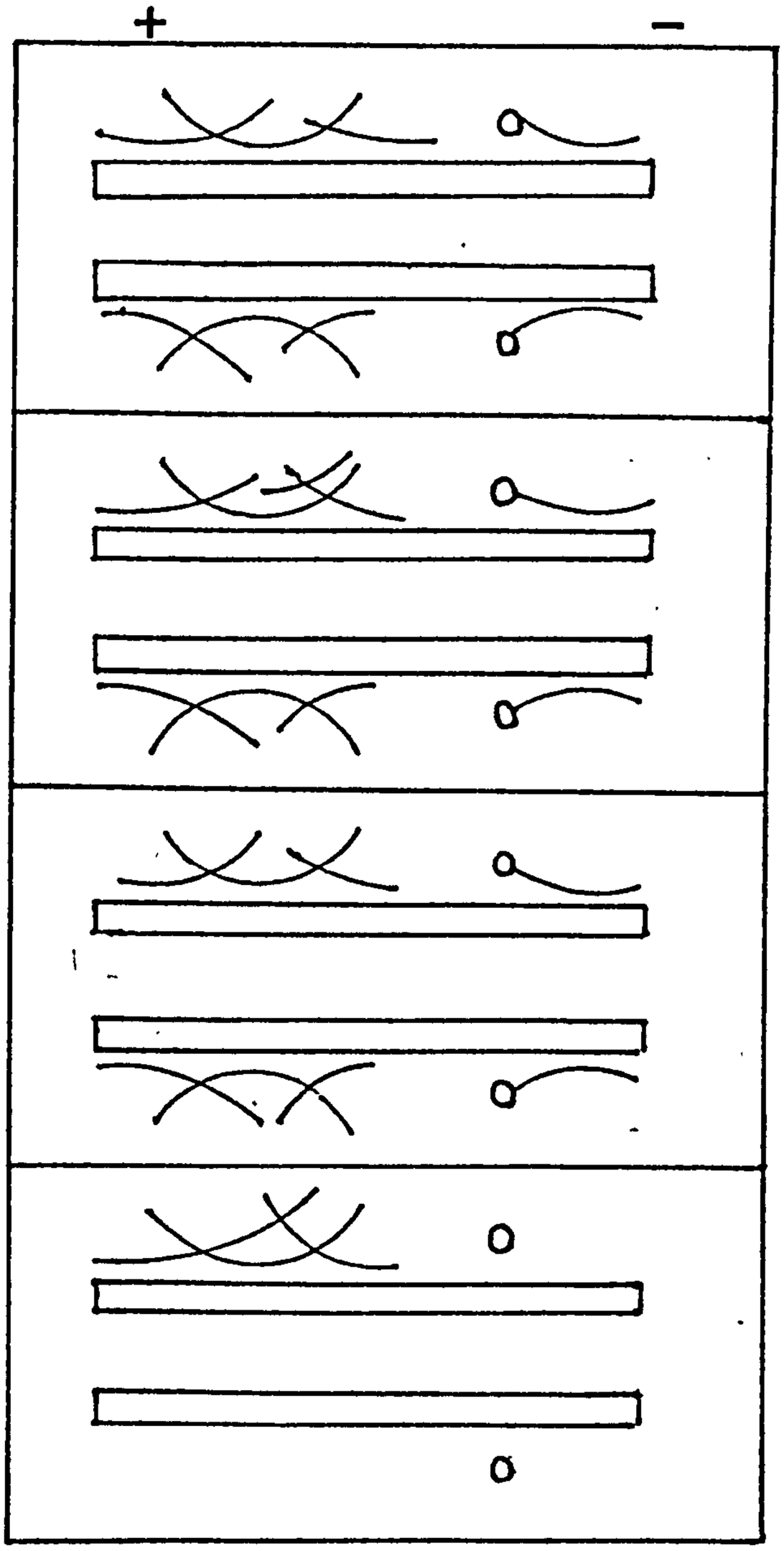
All serum samples taken from the above cases of nasal aspergillosis were positive for antibody to A. fumigatus with each of the 3 antigens used in D.D. and with antigen 121 which was used in C.I.E. tests.

The number of precipitation lines ranged from 1 to 3 in both D.D. and C. I. E. but the latter test was more sensitive and showed 3 lines in most of the serum samples (Table 29).

The pattern of arcs obtained by I.M.E. of 6 serum samples from case 1 are shown in Fig. 17. The 3rd serum sample, taken immediately after rhinotomy, was the only one in which 5 arcs were observed; 1 arc lay on the cathode side and the others on the anode side. Only 4 arcs were present in the other samples from this case. Test serum S2, from a case of nasal aspergillosis which had been cured, gave only 3 arcs on the anode side.

Nasal washings and/or swabs from the cases were examined. A swab from case 1, taken on the day before rhinotomy was performed, was positive on microscopic examination and A. fumigatus was obtained in culture. On 10.3.82 a swab proved negative on microscopic examination but a second swab taken 2 hours later was positive. The clinician had located the site of the lesion before taking the swab sample. Both swabs yielded A. fumigatus in culture but the difference between the results from the 2 swabs was dramatic (Fig. 18). Nasal washings taken 6 days after surgery were negative by microscopy and culture as were swabs taken on the 8th and 27th April.

A nasal swab from case 3, taken on admission of the dog, was negative by microscopy and culture; swabs and nasal washings taken on 12th May were negative on microscopic examination but yielded



Date	Titre
15.12.81	1/8
17.12.81	1/8
13. 1.82	1/2
22. 2.82	1/8
2. 3.82	1/4
4. 3.82	1/8
S2	
S.N.S.	

Fig. 17. Precipitation arc patterns after I.M.E. produced by serial serum samples from Case 1 and by S2 against antigen 121.

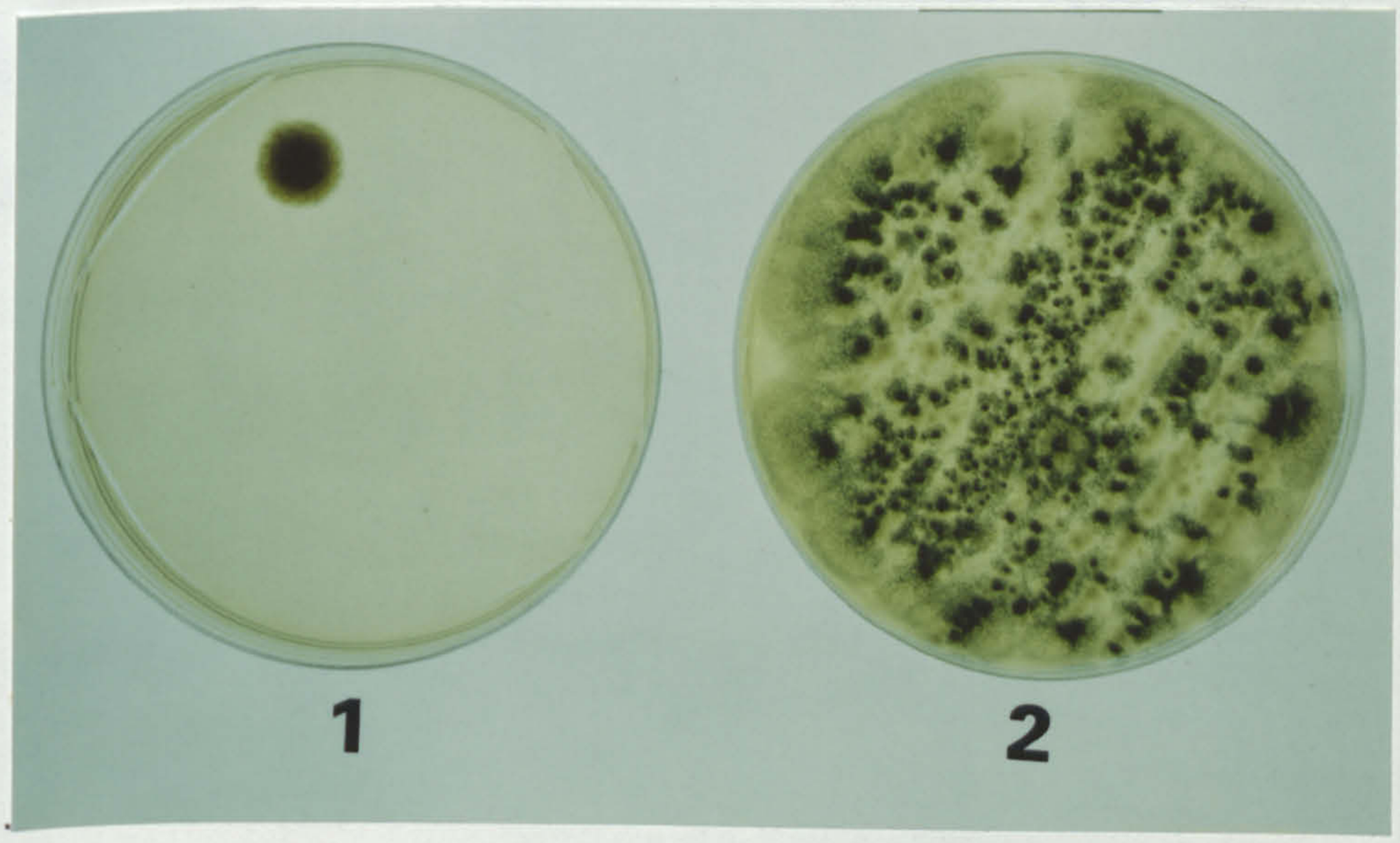


Fig. 18. Difference in culture results from swabs taken from the same dog on the same day:

1. Swab taken "blindly".
2. Swab taken after the lesion had been located by endoscopic examination.

A. fumigatus in culture.

Nasal washings were obtained from case 4 on the day after surgery. These proved negative by microscopy but gave A. fumigatus in culture.

A total of 26 cases from dogs suspected of having nasal aspergillosis, examined in the mycology laboratory by culture from nasal swabs and by serological tests were studied retrospectively. Ten of the 26 swabs were positive in culture, 6 giving A. fumigatus, 2 Penicillium sp., 1 A. flavus and 1 Aspergillus sp.. Of the 6 dogs from which A. fumigatus was obtained in culture, 5 were also positive in serological tests. Seven other serum samples showed antibody to A. fumigatus and this species was subsequently obtained in culture from swabs. Two cases, re-sampled several days later gave negative results on culture.

All serum samples from the 4 cases of nasal aspergillosis were positive in D.D. with each of the 3 antigens used (170, 121 and M1) and also in C.I.E. tests with 121; the latter test giving higher numbers of lines (Table 29). Titres by D.D. ranged from 0 to 1/32 and this high titre was found only in cases 2, 3 and 4. In titrations by C.I.E. a titre of 1/32 was found only once, in a serum sample from case 2. In the other samples titres ranged from 0 to 1/16. The results of titration by D.D. and C.I.E. agreed in 13 samples; D.D. gave higher titres in 7 samples and C.I.E. higher titres in 7.

It was noted that following rhinotomy the titre of the serum rose

but in samples taken immediately after exploratory surgery the titres dropped.

Table 29. The results from serum samples from the 4 cases of nasal aspergillosis tested and titrated against antigen 121 by D.D. and C.I.E.

Case	Date	Serum	Case history	Number of lines D.D.	Number of lines C.I.E.	Titre D.D.	Titre C.I.E.
1	15.12.81	1		2	3	1/8	1/4
	17.12.81	2		2	3	1/8	1/4
	13. 1.82	3	Rhinotomy & Th.	3	3	1/2	1/4
	22. 2.82	4		1	3	1/8	1/8
	2. 3.82	5	Keto.	1	3	1/4	1/8
	4. 3.82	6		1	3	1/8	1/4
	10. 3.82	7	Surgery	1	3	0	1/4
	17. 3.82	8		2	3	1/8	1/8
	18. 3.82	9		1	3	1/16	1/16
	24. 3.82	10		2	3	1/8	1/16
	7. 4.82	11		1	2	1/8	1/8
	10. 4.82		½ Keto.sent home				
	18. 4.82		Keto. stopped				
	26. 4.82	12		1	3	1/2	1/4
	11. 5.82	13		1	2	1/2	1/2
	7. 6.82	14		1	3	0	1/2
2	28. 4.82	1		2	1	1/2	1/2
	5. 5.82	2	Keto.	2	3	1/2	1/4
	11. 5.82		Rhinotomy				
	12. 5.82	3		2	3	1/4	1/4
	19. 5.82	4		2	3	1/32	1/16
	2. 6.82	5	Surgery	2	3	1/16	1/16
	4. 6.82	6	Destroyed	2	3	1/32	1/32
3	29. 4.82	1		2	3	1/32	1/16
	5. 5.82	2		2	3	1/32	1/8
	12. 5.82	3	Surgery,Keto.	2	2	1/8	1/8
	16. 5.82		Died				
4	11. 5.82	1	Keto.	2	3	1/16	1/16
	17. 5.82		Surgery				
	18. 5.82	2		3	3	1/8	1/8
	26. 5.82	3	Keto. stopped	3	3	1/32	1/16
	2. 6.82	4	Destroyed	1	3	1/8	1/8

Th. = Thiabendazole Keto. = Ketoconazole

DISCUSSION

As the lesions of canine nasal aspergillosis are well-developed before clinical signs of disease become evident and as no-one has attempted to reproduce the disease experimentally, little is known about the early stages in the pathogenesis of this condition. A. fumigatus, the main causal fungus, is widespread in nature and my serological surveys showed that antibody to this fungus was present in sera from dogs which were not affected by the disease, especially in those dogs from environments such as farms and stables in which A. fumigatus might be expected to be present in quantity.

Culture of normal turbinates and of nasal swabs, however, yielded few isolates of Aspergillus spp. which would suggest that usually exposure is low, or more probably, that the nasal defence mechanisms of a healthy dog are highly efficient. That this is so is suggested by the fact that only one case of pulmonary aspergillosis has been reported (Ohshima, 1979), and that disseminated aspergillosis is rare and is associated with severe underlying disease (Isoun, 1975; Poonacha and Smith, 1976).

In the healthy dog infection could result, as in other animal species, from an overwhelming exposure to the fungus or by disruption of the defence system by trauma or by some disease condition such as bacterial infection of the nasal passages or by some debilitating disease like cancer. Case 4, at autopsy, was found to have a

hepatoma.

The clinical picture of nasal aspergillosis in the 4 cases was similar, each showing nasal discharge, pain in the nose and at least one episode of epistaxis. These clinical signs have been reported by Spreull (1971), Cadwallader et al (1973) and Lane et al (1974) as those most often found in canine nasal aspergillosis. None of the cases showed a change in temperament or any other sign of involvement of the nervous system such as was described by Stazzi (1905), Soltys et al (1971), Parker et al (1971) and Cadwallader (1973).

Case 3 died suddenly without showing any sign of deterioration; it was bright and eating well on the day before it died. A complete post mortem examination was not possible because of advanced autolysis; the dog died on a Saturday night and the body was not put into the cold room. However, septate hyphae were seen on microscopy of the spinal fluid and A. fumigatus was isolated in pure culture. This would suggest that the infection had become generalised.

At autopsy, case 2 showed the typical picture of destructive invasion of the nasal passages by A. fumigatus. Apart from the nasal aspergillosis, this young dog was healthy and no underlying disease was detected. The lesions produced by the fungus were not limited to destruction of soft tissues but involved cartilage and bones also. Most important was the fact that a severe inflammatory reaction was preceding the invasion of the tissues. This observation

suggested the possibility that a toxic substance which had been released from the fungus had caused the inflammatory reaction. That A. fumigatus could produce toxin which had haemolytic and histiolytic properties and was also antigenic was reported by Henrici (1939). If an endotoxin of A. fumigatus was present it could also play a part in stimulating the production of antibody in the animal.

In case 4, an old dog, a liver problem was suspected when the drug used to treat the nasal aspergillosis was not tolerated. The dog was dull, inappetant and in pain and was destroyed. At autopsy, a severe necrotising rhinitis with destruction of the turbinates was found as was a tumour of the right medial lobe of the liver.

It was found that diagnosis of the disease ought to be an exercise in co-operation between the clinician and laboratory. However, the first step must be taken by the clinician, especially because the clinical signs, nasal discharge, epistaxis and pain in the nasal area can occur also with nasal tumour and in upper respiratory tract infections. Radiography and serology should therefore be carried out in all suspected cases. However, as my serological surveys showed that it is possible to obtain precipitating antibody in dogs free from nasal aspergillosis, I would suggest that serum samples from dogs with suspected nasal aspergillosis should be titrated to eliminate the possibility of a false positive. A second sample 15 days later would be advisable either as confirmation of a negative result or

if positive as the start of a follow-up of the case.

In this study, microscopy and culture of swabs and nasal washings gave varied results and as these may be false positive or false negative their use was not considered to be reliable. This was in accordance with the findings of Black et al (1973), Lane (1974) and Chandler (1975) who found that swabs from infected animals were negative in culture. The exception to this tendency of failure to obtain a good swab sample occurs when, with the help of an endoscope, the clinician is able to locate and sample the lesion. Culture was more reliable than microscopic examination.

In this survey it was found that Aspergillus spp. could be isolated from nasal turbinates of normal dogs. This showed that even if a culture is obtained from a nasal swab or washing, the diagnosis of aspergillosis must be based on other tests due to the lack of reliability of this teqhnique. Material obtained by surgery was best for confirming the diagnosis. With this material direct examination in KOH often enabled identification of the fungus as sporing heads are frequently present. Identification can then be confirmed by culture.

In the serological surveys it was found that antibody to A. fumigatus could be demonstrated by D.D. and C.I.E. in sera from dogs free from nasal aspergillosis. It was therefore decided to investigate the use of titration of the serum samples to reduce the

possibility of false positive results. It was found that titration of sera was not only a possible way of discarding false positives but it was of help in assessing the immunological changes in the infected dogs. It was noted that a high titre (1/32) was found in cases 2, 3 and 4 which were those that died or had to be destroyed.

D.D. and C.I.E. were found to be equally effective in detecting antibody in serum from cases. C.I.E. showed a higher number of lines in these samples. When titration results by both tests were evaluated, a discrepancy between them appeared. It was also noted that, contrary to the expected higher sensitivity of C.I.E., this test only once gave a titre of 1/32 while D.D. showed it in 5 cases. This finding suggested that D.D. would be the better test to use in titrating serum samples from the cases. C.I.E. also had the disadvantage of being more liable than D.D. to show antibody due to exposure.

I.M.E. showed that in one serum sample from case 1 an extra arc appeared, giving 5 arcs in all. In S2, serum from a dog which had nasal aspergillosis but at autopsy was found to be cured, only 3 arcs were present. The extra arcs in the serum sample from case 1 may, therefore, indicate the presence of active infection.

The results of the serological surveys showed that antibodies to A. fumigatus were present in some normal dogs and especially in dogs living in environments such as farms in which A. fumigatus is liable to be present in quantity. Isoun (1975) suggested that

veterinary practitioners should warn their clients of the dangers of allowing dogs to inhale or ingest mouldy food.

None of the cases seen came from farms, and cases 1 and 2 did not have any concurrent disease predisposing to infection. It is possible that factors such as trauma could be of importance especially in cases in young and healthy dogs.

The successful treatment of case 1 with ketoconazole encouraged the use of the drug in the other cases but, unfortunately, the results were unsatisfactory. In case 2 it failed to help the dog, either because the infection was too widely spread, or because the drug could not reach the fungus within the debris of necrotised tissues in which it was found growing. In case 4 the drug, which may adversely affect the liver, was not tolerated, probably because of concurrent hepatoma which may also have been a predisposing factor in the development of the aspergillosis as was suggested by Dawson et al (1973). It is obvious that therapy of canine nasal aspergillosis is still rather uncertain and much further investigation is required.

The use of ketoconazole in the treatment of canine nasal aspergillosis had not been attempted before. It was decided to use 40mg/Kg as recommended by Janssen Pharmaceutical Research Laboratories as the highest dose which can be used with safety. The failure of treatment in case 2 suggested the possibility of strain resistance. Isolates of A. fumigatus from each of the 4 cases were sent to the

Medical Mycology Unit of the University of Glasgow where Professor J. C. Gentles performed minimal inhibition concentration tests. The results from each were almost identical, proving that strain resistance was not the cause of the failure of the treatment. Thus, although the drug was active against A. fumigatus in vitro it was less effective in vivo.

CONCLUSIONS

In this study on antigens produced by A. fumigatus, it was found that the quality of the antigens produced by the 5 strains investigated was influenced by the strain used and the conditions under which it was cultured.

Strain V526 produced the best antigens. This strain, which was morphologically abnormal, was obtained from a case of canine nasal aspergillosis. Culture filtrate antigens prepared from this strain grown on G.P. liquid medium at 37°C were shown to be those with the best serological activity.

Both G.P. and Y.M., the media used in antigen production, proved satisfactory but some differences were found in the amount of protein in the antigens. Those produced on Y.M. showed a higher protein content than those produced on G.P.

The medium also affected the weight of mycelium produced. Of the 2 strains cultured on both Y. M. and G.P. both produced a lighter weight of mycelium on Y.M.

The weight of mycelium produced varied from strain to strain. V526 yielded the lowest and A1944 and V539 the heaviest mycelium. Culture filtrate antigens of V526 were ranked best and those of A1944 and V539 worst. It would therefore appear that the best culture filtrate antigens are associated with low weight of mycelium.

For indi idual strains it was noted that the temperature of incubation affected the weight of mycelium produced; the higher the

temperature, the heavier the weight of the mycelium.

It was noted that the ability of the various antigens to react in precipitation tests with animal sera was influenced by whether the antigen had been made from culture filtrate or from mycelium. In D.D. tests mycelial antigens proved better than culture filtrate antigens but in C.I.E. tests the reverse was the case.

Temperature of incubation also affected antigenic ability; 37°C and 28°C were both suitable for antigen production but 46°C gave reduced antigenic ability.

The age of the culture was also important. This was clearly shown by the failure of young culture filtrate antigens to react with S1, the "exposure" serum, while the older antigens of the same group and also the mycelial antigens did react. With S2 and S3, sera from cases of disease, culture filtrate and mycelial antigens showed only minor differences. This suggests that young culture filtrate antigens might be of use in differentiating between antibody due to exposure from that due to infection.

Overall, D.D. tests gave a higher percentage of positive results (88.4%) than C.I.E. (77%). S1 showed a higher percentage of positive tests in D.D. than it did in C.I.E. while S2 and S3 showed very similar results in both tests. Culture filtrate antigens proved less effective than mycelial antigens with the 3 test sera in D.D.. In C.I.E. tests however, mycelial antigens were less good than culture filtrate antigens with S1 but gave better results with S2 and S3.

All antigens were titrated by D.D. using S3. It was noted that culture filtrate antigens tended to give higher titres than mycelial antigens. Titre, however, did not correlate with antigenic ability to any great extent because of this difference between the different types of antigens.

It was shown that fungal exudates make very good antigens. Exudates from A. fumigatus, A. nidulans, A. terreus and A. ochraceus were collected and used unconcentrated in D.D. tests with their relevant immune serum raised in rabbits. All gave positive results. Exudates of A. nidulans and A. fumigatus also reacted well with sera from other animals with good results.

The factors involved in the production of exudates were investigated and it was shown that high humidity was important and that low amounts of sugars in the culture medium gave better yields.

Another original method of obtaining antigen was the use of the enhancing effect of allantoic fluid on the germination of spores of A. fumigatus. This antigen in C.I.E. tests with sera from cases of bovine mycotic abortion gave better results than those obtained with the routine antigens.

Serological tests were found to be unsatisfactory for diagnosis of mycotic abortion. Although antibody was detected in the serum of some cows which had aborted because of A. fumigatus infection it was not present in all. Antibody to A. fumigatus was shown to be present in serum from cows aborting due to infection by other species of fungus

and in sera from normal cattle. The amount of antibody to A. fumigatus in sera from cases of abortion caused by this species was shown to be low and it seems possible that the antibody detected may due to exposure rather than to the infection of the uterus.

In the serological survey it was shown that precipitating antibody to A. fumigatus was present in a small (4.2%) proportion of sera from healthy cats in the Glasgow area.

In 2 surveys, carried out in the same area on canine sera, 14.6% and 31.4% were shown to be positive for antibody to A. fumigatus.

In conjunction with the second serological survey, the fungal flora of the nasal antrum and turbinates of the dogs was investigated. Turbinates yielded higher numbers of fungi than did the nasal antrum. Geotrichum, Penicillium and Rhodotorula were the genera most frequently isolated. The number of isolates of Aspergillus spp. was lower than expected. The most important aspect of this survey was that it proved that antibody to A. fumigatus can occur in sera from healthy dogs.

Clinical signs of otitis externa were present in 22 of the above dogs. On culture one gave Candida sp. and the remainder P. pachydermatis. Serum antibody to this yeast, not previously reported, was demonstrated in 2 cases by D.D. Neither of these sera were positive when tested with A. fumigatus antigens, suggesting that in D.D. these 2 species are unlikely to give cross reactions.

The reliability of the methods used in laboratory diagnosis of

canine nasal aspergillosis were investigated. It was shown that microscopic examination of swab samples and of nasal washings, the materials most frequently sent to the laboratory by veterinary prectitioners, was unsatisfactory because on some occasions samples from cases known to be positive gave negative results. Culture of swabs and nasal washings proved better but, again, these were not completely reliable as false positive and false negative results were found. The only materials found to give completely reliable results were swab samples taken by a clinician who first located the site of the lesion with an endoscope and tissues removed from the turbinates or sinuses at operation.

It has been generally assumed that the presence of precipitating antibody to A. fumigatus in canine serum samples is diagnostic but the present study has shown that this is not so. A proportion of healthy dogs, especially those living in environments such as farms and stables where exposure to the fungus is likely to be high, have antibody. Titration of serum is suggested as the best way of differentiating antibody due to exposure from that due to infection.

Four cases of canine nasal aspergillosis admitted to the Veterinary Hospital were followed in detail. Treatment of the condition with ketoconazole was attempted. One dog was cured, one could not tolerate the drug because it had a hepatoma and in 2

cases treatment failed. Sections of lesions showed that small fungal colonies were completely surrounded by necrotic debris which would have prevented the drug from reaching the mycelium.

All cases were periodically investigated by microscopy and culture and serial serum samples were studied. Titration of serum samples by D.D. was shown to be the best method of assessing the progress of the dog. Steadily falling titres were found only in the case which recovered.

D.D. and C.I.E. which were used in these studies were both suitable for detecting antibody in the samples. D.D. was shown to be better for practical routine work as it is easy to perform and simple to interpret. C.I.E., although it gives results more quickly, requires special equipment, is more difficult to read and interpret and tends to show more false positives.

These studies have emphasised the need for close co-operation between the clinician and the laboratory worker in the diagnosis and treatment of fungal disease in animals.

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